

Practitioner's Docket No. 55729

PATENT

TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE (DO/US)  
ENTRY INTO THE U.S. NATIONAL STAGE UNDER CHAPTER I

PCT/JP00/04720 14 July 2000 15 July 1999  
INTERNATIONAL APPLICATION NO. INTERNATIONAL FILING DATE PRIORITY DATE CLAIMED

NOVEL NUCLEIC ACID BASE PAIR  
TITLE OF INVENTION

Ichiro HIRAO, Masahide ISHIKAWA, Tsuyoshi FUJIHARA and Shigeyuki YOKOYAMA  
APPLICANT(S)

Box PCT  
Assistant Commissioner for Patents  
Washington D.C. 20231  
ATTENTION: DO/US

NOTE: The completion of those filing requirements that can be made at a time later than 20 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 U.S.C. § 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase. See 37 C.F.R. § 1.491, which states: "An international application enters the national stage when the applicant has filed the documents and fees required by 35 U.S.C. § 371(c) within the periods set forth in § 1.494 and § 1.495."

WARNING: Where the items are those that can be submitted to complete the entry of the international application into the national phase subsequent to 20 months from the priority date, the application is still considered to be in the international stage. And if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (because international application papers are not covered by an ordinary certificate of mailing, 37 C.F.R. § 1.8(2)(ii)).

WARNING: Documents and fees must be clearly identified as a submission to enter the national stage under 35 U.S.C. § 371, otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

WARNING: Failure to pay the national fee within 20 months from the priority date will result in the abandonment of the application. The time for payment of the basic fee is not extendable. M.P.E.P. § 1893.01(a)(1), 6th ed., rev. 3.

CERTIFICATION UNDER 37 C.F.R. § 1.10\*  
(Express Mail label number is mandatory.)  
(Express Mail certification is optional.)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date March 15, 2001, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EL835032266US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Deanna M. Rivemider  
(type or print name of person mailing paper)

*Deanna Rivemider*  
Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

\*WARNING: Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing, 37 C.F.R. § 1.10(b).  
"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

1. Applicant herewith submits to the United States Designated Office (DO/US) the following items under 35 U.S.C. 371:

- a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).
- b. ☒ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and
- ☒ other fees (37 C.F.R. § 1.492), as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
*	TOTAL CLAIMS	53 - 20 =	33	x\$ 18.00=	\$594.00
	INDEPENDENT CLAIMS	7 - 3 =	4	x\$ 80.00=	\$320.00
	MULTIPLE DEPENDENT CLAIMS(S) (if applicable) + \$270.00				\$270.00
	The international search fee, as set forth in § 1.445(a)(2) to be paid to the US PTO acting as an international Searching Authority:				\$ 860.00
BASIC FEE**	<input type="checkbox"/> has been paid (37 CFR 1.492(a)(2)).....\$760.00 <input type="checkbox"/> has not been paid (37 CFR 1.492(a)(3)).....\$970.00				
	<input checked="" type="checkbox"/> where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) .....				\$860.00
	Total of above Calculation				= \$2,044.00
	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed also. (note 37 CFR 1.9, 1.27, 1.28)				-
	Subtotal				\$2,044.00
	Total National Fee				\$2,044.00
SMALL ENTITY	Fee for recording the enclosed assignment document \$40.00 (37 CFR 1.21(h)). (See Item 10 below). See attached "ASSIGNMENT COVER SHEET (37 CFR 3.34)".				\$
	TOTAL				Total Fees enclose \$2,044.00

**\*\*WARNING:** "To avoid abandonment of the application, the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 20 months from the priority date; \*\*\* (2) the basic national fee (see § 1.492(a)). The 20-month time limit may not be extended." 37 C.F.R. § 1.494(b).

- i. ☒ A check in the amount of \$ 2,044.00 to cover the above fees is enclosed.
- ii. ☐ Please charge Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_.  
A duplicate copy of this sheet is enclosed.

**WARNING:** If the translations of the international application and/or oath or declaration have not been submitted by the applicant within twenty (20) months from the priority date, the applicant will be so notified and given a period of time within which to file the translation and/or oath or declaration in order to prevent abandonment. The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than twenty (20) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than twenty (20) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 will apply. 37 C.F.R. § 1.494(c).

3. A copy of the International application as filed (35 U.S.C. § 371(c)(2)):
- a. ☐ is transmitted herewith.
  - b. ☐ is not required, as the application was filed with the United States Receiving Office.
  - c. ☒ has been transmitted
    - i. ☒ by the International Bureau. Date of mailing of the application From form PCT/IB/308): 25 January 01.
    - ii. ☐ by applicant on \_\_\_\_\_.
- Date

**NOTE:** Section 1.494(b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 20 months from the priority date to avoid abandonment. "The International Bureau nominally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies the applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage and applicant has received notice from the International Bureau, applicant need only pay the basic national fee by 20 months from the priority date." [This can now be paid subsequently with a surcharge.] Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35.

4. A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):
- a. ☒ is transmitted herewith.
  - b. ☐ is not required as the application was filed in English.
  - c. ☐ was previously transmitted by applicant on \_\_\_\_\_.
- Date

5. ☐ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

**NOTE:** The Notice of January 7, 1993 indicates that 37 C.F.R. § 1.494(d) was "amended to clarify the existing practice that PCT Article 19 Amendments must be submitted by 20 months from the priority date, which time may not be extended." This Notice further advises: "Of course, the failure to do so does not result in loss of the subject matter of PCT Article 19 amendments. The applicant may submit that subject matter in a preliminary amendment filed under Section 1.121. In many cases, filing an amendment under Section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 35. See item 11(c) below. See also 37 C.F.R. § 1.494(d).

- a. ☐ are transmitted herewith.
  - b. ☐ have been transmitted
    - i. ☐ by the International Bureau. Date of mailing of the amendment (from form PCT/IB/308): \_\_\_\_\_.
    - ii. ☐ by applicant on \_\_\_\_\_.
- Date
- c. ☒ have not been transmitted, as
    - i. ☐ no notification has been received that the International Search Authority has received the Search Copy.

- ii ☐ the Search Copy was received by the International Searching Authority, but the Search Report has not yet been issued. Date of receipt of Search Copy from form PCT/ISA/202): \_\_\_\_\_.
- iii. ☒ applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210): 17/10/00.
- iv. ☐ the time limit for the submission of amendments has not yet expired. The amendments, or a statement that amendments have not been made, will be transmitted before the expiration of the time limit under PCT Rule 46.1.
6. ☒ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)):
- a. ☐ is transmitted herewith.
- b. ☐ is not required as the amendments were made in the English language.
- c. ☒ has not been transmitted for reasons indicated at point 5(c) above.
7. ☒ An oath or declaration of the inventor including power of attorney (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115
- a. ☐ was previously submitted by applicant on \_\_\_\_\_  
Date
- b. ☐ is submitted herewith, and such oath or declaration
- i. ☐ is attached to the application.
- ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or (c) and 5(b); and states that they were reviewed by the inventor, as required by 37 C.F.R. § 1.70.
- iii. ☒ will follow.
- II. Other document(s) or information included:
8. ☒ An international Search Report or Declaration under PCT Article 17(2)(a):
- a. ☒ is transmitted herewith.
- b. ☐ has been transmitted by the International Bureau. Date of mailing from form PCT/IB/308): \_\_\_\_\_.
- c. ☐ is not required, as the application was searched by the United States International Searching Authority.
- d. ☐ will be transmitted promptly upon request.
- e. ☐ has been submitted by applicant on \_\_\_\_\_  
Date
- f. ☐ is not transmitted, as the international search has not yet issued.
9. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:
- a. ☒ is transmitted herewith.  
Also transmitted herewith is (are)  
☒ Form PTO-1449 (PTO/SB/08A and 08B)  
☒ Copies of citations listed
- b. ☐ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).
- c. ☐ was previously submitted by applicant on \_\_\_\_\_  
Date

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10. ☐ An assignment document is transmitted herewith for recording. A separate  
☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW  
☐ PATENT APPLICATION" or  
☐ FORM PTO—1595 is also attached.  
Please mail the recorded assignment document to:  
i. ☐ the person whose signature and address appears below.  
ii. ☐ the following:
11. ☒ Additional documents  
a. ☒ Copy of request (PCT/RO/101)  
b. ☒ International Publication No. WO 01/05801 A1  
i. ☒ Specification, claims and drawing  
ii. ☐ Front page only  
c. ☐ Preliminary amendment (37 C.F.R. § 1.121)  
d. ☒ Other: PCT Request Form  
Form PCT/IB/308 and PCT/ISA/202  
Form PCT/IB/301 and PCT/IB/304
12. ☒ The above checked items are being transmitted  
a. ☐ before the 18th month publication.  
b. ☒ after publication and the article 20 communication, but before 20 months from  
the priority date.  
c. ☐ after 20 months (revival).

NOTE: *Petition to revive (37 C.F.R. § 1.137(a) or (b)) is necessary if 35 U.S.C. § 371 requirements are submitted after 20 months.*

13. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant  
on \_\_\_\_\_ namely:  
Date

#### AUTHORIZATION TO CHARGE ADDITIONAL FEES

**WARNING:** *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: *"A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).*

NOTE: *"Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).*

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[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 04-1105.

[X] 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

**WARNING:** Because failure to pay the national fee within 20 months without extension (37 C.F.R. § 1.494(b)(2)), results in abandonment of the application, it would be best to always check the above box.

[X] 37 C.F.R. § 1.492(b), (c), and (d) (presentation of extra claims)

**NOTE:** Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment, prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

[X] 37 C.F.R. § 1.17 (application processing fees)

[X] 37 C.F.R. § 1.17(a)(1)-(5) (extension fees pursuant to § 1.136(a)).

[ ] 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b)).

**NOTE:** Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

**NOTE:** 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying or at the time of paying . . . issue fee. . ." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[X] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 20 months after the priority date).



SIGNATURE OF PRACTITIONER

Reg. No. 33,860

Peter F. Corless

(type or print name of practitioner)

Tel. No.: (617) 523-3400

EDWARDS & ANGELL, LLP

Dike, Bronstein, Roberts & Cushman, IP Group

130 Water Street

P.O. Address

Customer No.:

Boston, Massachusetts 02109



13 Rec'd PST/PTO 16 JUL 2001  
09/787196

Docket No. 55729 (17526)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

APPLICANT: I. Hirao, et al.  
SERIAL NO.: 09/787,196 EXAMINER: not yet assigned  
FILED: March 15, 2001 GROUP: not yet assigned  
FOR: NOVEL NUCLEIC ACID BASE PAIR

Assistant Commissioner of Patents  
and Trademarks  
Washington, DC 20231

Sir:

**AMENDMENT IN RESPONSE TO NOTICE TO COMPLY  
WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING  
NUCLEOTIDE SEQUENCE AND/OR AMINO ACIDS SEQUENCE DISCLOSURES**

In response to the Notice to Comply with Requirements for Patent Applications  
Containing Nucleotide Sequence and/or Amino Acids Sequence Disclosures mailed May 11,  
2001, please amend the above-identified application as follows:

**In the Specification:**

Page 17, line 13, after "Template 3:" insert --(SEQ ID NO: 1)--;  
line 14, after "Template 5:" insert --(SEQ ID NO: 2)--;  
line 15, after "Template 6:" insert --(SEQ ID NO: 3)--;  
line 16, after "Template 7:" insert --(SEQ ID NO: 4)--;  
line 17, after "Template 8:" insert --(SEQ ID NO: 5)--;  
line 18, after "Template 9:" insert --(SEQ ID NO: 6)--;  
line 20, after "Template 1:" insert --(SEQ ID NO: 7)--;  
line 21, after "Template 2:" insert --(SEQ ID NO: 8)--;

line 22, after "Template 4:" insert --(SEQ ID NO: 9)--.

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Page 18, line 2, after "Primer 1" insert --(SEQ ID NO: 13)--;  
 line 3, after "Primer 2" insert --(SEQ ID NO: 14)--;  
 line 4, after "Primer 3" insert --(SEQ ID NO: 15)--;  
 line 13, after "Primer 1" insert --(SEQ ID NO: 13)--;  
 line 14, after "Template 1:" insert --(SEQ ID NO: 7)--;  
 line 16, after "Primer 1" insert --(SEQ ID NO: 13)--;  
 line 17, after "Template 3:" insert --(SEQ ID NO: 1)--.

Page 19, line 7, after "Primer 2" insert --(SEQ ID NO: 14)--;  
 line 8, after "Template 1:" insert --(SEQ ID NO: 7)--;  
 line 10, after "Primer 2" insert --(SEQ ID NO: 14)--;  
 line 11, after "Template 2:" insert --(SEQ ID NO: 8)--;  
 line 13, after "Primer 2" insert --(SEQ ID NO: 14)--;  
 line 14, after "Template 3:" insert --(SEQ ID NO: 1)--.

Page 21, line 4, after "Coding strand" insert --(SEQ ID NO: 10)--;  
 line 5, after "Template 1-3" insert --(SEQ ID NOS 1, 7 or 8)--.

Page 22, line 1, after "coding strand;" insert --(SEQ ID NO: 10)--;  
 line 2, after "template;" insert --(SEQ ID NOS 1, 7 or 8)--;  
 line 4, after "GpGpGpApGpGpApApGpApNpApGpApGpCpA" insert --(SEQ ID NO: 11)--.

Page 24, line 13, after "Primer" insert --(SEQ ID NO: 12)--;  
 line 14, after "Template" insert --(SEQ ID NOS 1, 7 or 8)--.

Page 25, line 13, after "ATAATACGACTCACTATAGGG" insert --(SEQ ID NO: 10)--;  
 line 14, after "template" insert --(SEQ ID NOS 1, 7 or 8)--;



line 20, after "r(GGG\*AGG\*A\*AG\*An\*AG\*AGC\*A)" insert --(SEQ ID NO: 11)--.

Page 26, line 1, after "formula:" insert --(SEQ ID NO: 11)--.

Page 44, line 4, after "Primer 1:" insert --(SEQ ID NO: 13)--;

line 5, after "Primer 2:" insert --(SEQ ID NO: 14)--;

line 6, after "Primer 3:" insert --(SEQ ID NO: 15)--;

line 7, after "Template 1:" insert --(SEQ ID NO: 7)--;

line 8, after "Template 2:" insert --(SEQ ID NO: 8)--;

line 9, after "Template 3:" insert --(SEQ ID NO: 1)--;

line 10, after "Template 4:" insert --(SEQ ID NO: 9)--;

line 11, after "Template 5:" insert --(SEQ ID NO: 2)--;

line 12, after "Template 6:" insert --(SEQ ID NO: 3)--;

line 13, after "Template 7:" insert --(SEQ ID NO: 4)--;

line 14, after "Template 8:" insert --(SEQ ID NO: 5)--;

line 15, after "Template 9:" insert --(SEQ ID NO: 6)--.

#### REMARKS

Responsive to the Office Communication, Applicants submit herewith 5 sheets of Sequence Listing to include as part of this Application. Applicants also submit herewith a statement that the context of the substitute sheets and computer readable copies are the same.

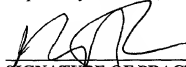
#### STATEMENT UNDER 37 CFR §1.825(a)

I hereby state that the substitute sheets, which include the Sequence Listing are supported

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1052410 96128260

in the Application as filed. I hereby state that the substitute sheets do not include new matter.

Respectfully submitted,



**SIGNATURE OF PRACTITIONER**

Reg. No. 33,860

Tel. No. 617-439-4444

Peter F. Corless

*(type or print name of practitioner)*

EDWARDS & ANGELL LLP

P.O. Box 9169

Boston, MA 02209

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 55729

re patent application of

HIRAO, ICHIRO et al.

Serial No. 09/787,196

Filed: March 15, 2001

For: NOVEL NUCLEIC ACID BASE PAIR

STATEMENT TO SUPPORT FILING AND SUBMISSION IN  
ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents  
Washington, D.C. 20231  
**Box SEQUENCE**

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and

3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

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States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

Date

**HARBOR CONSULTING**

Intellectual Property Services  
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Suite 262  
Portsmouth, N.H.  
800-318-3021

James A. Coburn

NOVEL NUCLEIC ACID BASE PAIRField of Invention

The present invention relates to formation of selective novel artificial nucleic acid base pair by utilizing steric hindrance.

The present invention further relates to replication and transcription of nucleic acid using the novel artificial nucleic acid base pair of the present invention, and a system for protein synthesis or functional nucleic acid. More particularly, the present invention pertains the novel artificial nucleic acid having properties to form selective base pair by applying steric hindrance, preferably to form selective base pair by applying steric hindrance, electrostatic repulsive force or stacking action, a process for production thereof, codon containing the same, nucleic acid molecule containing the same, a process for production of novel protein using the above nucleic acid molecules or non-natural gene.

Background Art

Genetic information of organisms in the earth are transferred by using nucleic acids comprising of four bases consisting of adenine (A), guanine (G), cytosine (C) and thymine (T) as a gene. Proteins are synthesized according to genetic informations of mRNA which is transcribed from DNA of gene. In that occasion, 64 types of codon consisting of 3 bases ( $4^3 = 64$ ) correspond to 20 types of amino acids.

If novel nucleic acid base (X and Y, in which X and Y form specific base pair) can be created in addition to already known for bases (A, G, C, T),

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numbers of codon can be increased greatly ( $6^3 = 216$ ). As a result, proteins containing non-natural amino acids can possibly be synthesized by matching the newly created codons with non-natural amino acids [J. D. Bain, et al. Nature, 356, 537-539 (1992)].

Heretofore, a pair of isocytosine and isoguanine has been reported as an artificial base pair except for A-T and G-C. Isoguanine tends to form base pair with thymine due to tautomerism of isoguanine [C. Switzer, et al. J. Am. Chem. Soc. 111, 8322-8323 (1989); C. Y. Switzer, et al. Biochemistry 32, 10489-10496 (1993)]. Several novel base pairs have been reported, but there were problems on recognition by polymerase and no practical use has known [J. A. Piccirilli, et al., Nature, 343, 33-37 (1990); J. Horlacher, et al. Proc. Natl. Acad. Sci. USA, 92, 6329-6333 (1995); J. C. Morales, et al., Nature struct. biol., 5, 954-959 (1998)].

Nucleic acid molecules having various functions were found by in vitro selection method [A. D. Ellington, et al. Nature 346, 818-822 (1990); C. Tuerk, et al. Science 249, 505-510 (1990)]. If the novel base pair X-Y hereinabove can be recognized by polymerases such as DNA polymerase, RNA polymerase and reverse transcriptase, the present in vitro selection method using 4 bases can be performed by using 6 bases, then possibility to create nucleic acid molecules having novel function, which could not be practically realized by using 4 bases, can be expected.

Further, creation of novel base pair has expected for treatment of hereditary diseases caused by gene abnormality, in which one or more base in the gene is replaced by different base.

We have studied extensively to created novel artificial nucleic acid

base pairs, which could not form base pair with natural nucleic acid, but could selectively form base pair by themselves and could be recognized by various polymerases. We have found that formation of nucleic acid base pair could be inhibited by applying steric hindrance of nucleic acid base, and formation of selective base pair between newly designed nucleic acid bases could be made. Further, we have found that such the newly designed nucleic acids could be recognized by various natural polymerases.

For example, in order not to form base pair with thymine but to form steric hindrance with keto group at position-6 of thymine, 2-amino-6-(N,N-dimethylamino) purine (hereinafter designates as X), in which two bulky methyl groups are introduced in amino group at position-6 of 2,6-diaminopurine, is designed. As a result, the X does not form base pair with thymine, but bases such as pyridine-2-one (hereinafter designates as Y), an analog of thymine, in which oxo group at position-6 is replaced by hydrogen atom, can form base pair with X (refer to Fig. 1).

Further, we have synthesized DNA oligomer containing 2-amino-6-(N,N-dimethylamino)-9-(2'-deoxy- $\beta$ -D-ribofuranosyl) purine (hereinafter designates as dX) and 3-(2'-deoxy-5'-triphosphoro- $\beta$ -D-ribofuranosyl) pyridine-2-one (hereinafter designates as dYTP), and found that dYTP or its ribonucleotide (rYTP) could be incorporated selectively into DNA or RNA as a complementary strand of the above dX.

This compound could hinder base pairing with natural base such as thymine (or uridine) (refer to Fig. 2 b) and cytosine in some extent due to steric bulkiness of dimethylamino group in the base (dx in Fig. 2 a). However, this steric hindrance could affect to the neighboring bases, and simultaneously

could give inferior effect on stacking between bases, and resulted low rate of incorporation of dYTP by Klenow fragment as well as insufficient suppression for incorporation of thymidine triphosphate (dTTP) to dx.

We have examined novel artificial base pair by considering not only steric hindrance but also electrostatic repulsion between bases and stacking action with the neighboring bases, and could obtain artificial base pair with superior selectivity.

#### Disclosure of Invention

The present invention provides ideas for selective formation of novel artificial nucleic acid base pair as a result of recognition of base pairing by polymerase such as DNA polymerase by utilizing steric hindrance between base pairs, preferably be generating steric hindrance between only base pair plane without giving deterioration for stacking between bases and more preferably by selecting bases utilizing electrostatic repulsion against natural bases.

An aspect of the present invention is to provide novel artificial nucleic acid base pair which does not form base pair with natural nucleic acid and forms selective base pair in themselves as well as being recognized by various polymerases. Further aspect of the present invention is to provide artificial nucleic acid, codon containing the same, nucleic acid molecule, non-natural gene and application thereof.

The present invention relates to a method for constructing selective base pair comprising introducing a group having ability to form steric hindrance, preferably a group having ability to form steric hindrance and



electrostatic repulsion, and stacking action in nucleic acid base. More particularly, the present invention relates to a method for constructing selective base pair wherein the said group having ability to form steric hindrance is a group to hinder formation of base pair with base part of natural nucleic acid, to hinder formation of base pair with base part of natural nucleic acid by an action of said steric hindrance and electrostatic repulsion, and to form stable structure with neighboring bases by the stacking action, and the said base pair can be recognized by polymerase.

Further, the present invention relates to a method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance in the nucleic acid base part, preferably utilizing steric hindrance and electrostatic repulsion, and stacking action in the nucleic acid base part. More particularly, the present invention relates to a method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance, preferably hindering to construct base pair with the natural nucleic acid base part by utilizing steric hindrance and electrostatic repulsion and stabilizing with the neighboring bases by the stacking action, and the said base pair can be recognized by polymerase.

The present invention relates to a nucleic acid, which can construct selective base pair, prepared by introducing a group having ability to form steric hindrance, preferably a group having ability to form steric hindrance and electrostatic repulsion, and stacking action in nucleic acid base. More particularly, the present invention relates to a nucleic acid for constructing selective base pair wherein the said group having ability to form steric hindrance, preferably having ability to form steric hindrance and electrostatic

repulsion is to hinder formation of base pair with base part of natural nucleic acid, and more preferably to stabilize with the neighboring bases by the stacking action, and the said base pair can be recognized by polymerase.

The present invention discloses novel artificial nucleic acid which have similar behavior with nucleic acids containing natural bases and a method for designing such the nucleic acid. The nucleic acid of the present invention can be applied in the similar manner as the natural nucleic acid.

Consequently, the present invention relates to various applications using the nucleic acid of the present invention or nucleic acid designed by the method of the present invention.

More particularly, the present invention relates to a codon comprising one or more nucleic acid designed by the nucleic acid of the present invention or nucleic acid designed by the method of the present invention. The said codon can encode amino acids in the similar manner as the natural nucleic acid. The said amino acids can be non-natural amino acids. Further, the present invention relates to a nucleic acid molecule containing the nucleic acid of the present invention, the nucleic acid designed by the method of the present invention or the nucleic acid of the natural origin. The said nucleic acid molecule can encode proteins in the similar manner as the natural nucleic acid. Further the said nucleic acid molecule can maintain whole or part of genetic informations of the natural gene. Nucleic acid having complementary strand can be prepared by an action of various polymerases on such the nucleic acid molecule. The present invention also relates to such the process for production of complementary strands.

In addition, the nucleic acid of the present invention or the nucleic acid

designed by the method of the present invention can be introduced or substituted to a part of natural gene. Consequently, the present invention relates to a process for production of non-natural gene comprising introducing or substituting one or more nucleic acid of the present invention or the nucleic acid designed by the method of the present invention into the natural gene. The introduction or substitution can be performed with the codon unit of the present invention as described hereinbefore.

Further, the present invention relates to a process for production of protein having amino acid sequence based on codons of the non-natural gene or the nucleic acid of the present invention. Protein to which non-natural amino acid is introduced or substituted in the part of natural protein can be produced in case that codon containing the nucleic acid of the present invention or the nucleic acid designed by the method of the present invention encodes non-natural amino acid.

Consequently, the present invention provides a process for production of novel protein comprising substituting or introducing other natural or non-natural amino acid, preferably non-natural amino acid in a part of natural protein by the method of the present invention. According to this method, functions of amino acids in the protein coded by natural gene can be screened. The present invention also relates to a method for screening function of each amino acid of protein encoded by natural gene.

The present invention also relates to a microorganism transformed by non-natural gene containing the nucleic acid of the present invention or nucleic acid designed by the method of the present invention (hereinafter simply designates as the nucleic acid of the present invention).

Further, since the novel base pair of the present invention does not constitute base pairing with the natural bases, it is useful for treatment of hereditary diseases caused by gene in which one or more base is replaced by other base. The present invention provides pharmaceutical composition comprising novel base pair or a base in the said base pair.

An object of the present invention is to provide artificial nucleic acid which does not form base pair with a base of the natural nucleic acid and can be recognized by polymerase. The conventional artificial nucleic acid has produced by attempting to change at the position of hydrogen bond, as a result, base pairing with a base of the natural nucleic acid could not be hindered substantially as well as showing insufficient base pair selectivity. We have solved such the problem by introducing a group forming the steric hindrance, preferably by introducing a group forming steric hindrance and electrostatic repulsion and having stacking action. The present invention provides novel artificial nucleic acid which can form selective base pairing with artificial nucleic acids themselves.

Consequently, the present invention will be explained more concretely by referring examples hereinbelow, but these examples are illustrated only for the purpose of better understanding of the present invention, and the fact that the present invention is not limited by these examples is obvious according to the technical idea of the present invention explained hereinbefore.

A group which forms steric hindrance in the base part of the nucleic acid of the present invention can be a group only hinder hydrogen bonding with deteriorated base, and is not limited if it does not deteriorate for the properties as a base of nucleic acid. Preferably, the size thereof may be not to hinder

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formation of base pairs of other bases in the nucleic acid sequence. In addition, it is preferable not to have polar group and activated hydrogen atom, but is not necessary to consider if these polar group and activated hydrogen atom are located in the positions having distance impossible to form hydrogen bonding

Examples of group which forms steric hindrance are, for example, lower alkyl group such as ethyl, isopropyl, isobutyl or t-butyl group, preferably branched lower alkyl group, lower alkoxy group consisting of methyl or these lower alkyl groups, di-lower alkylamino group substituted by methyl or these lower alkyl groups and silyl group substituted by methyl or these lower alkyl groups.

Conventional chemical synthesis can be applied for methods of introducing groups to form steric hindrance in the base.

Examples of groups having actions for steric hindrance, electrostatic repulsion and stacking action on base part of the nucleic acid are group having steric hindrance which hinders hydrogen bonding having deteriorated action between bases, having electrostatic repulsive force and having  $\pi$  electron for stacking action. These groups are not limited if they have deteriorating actions as a nucleic acid base. More preferably, a group having size not to hinder base pairing for other nucleic acid is preferable. Further, groups without having polar site for hydrogen bond and activated hydrogen atom are preferable, however if these polar site or activated hydrogen is located at distal position where hydrogen bonding may be impossible, it may not necessary to consider.

Examples of groups having steric hindrance, electrostatic repulsion and stacking action in the base pair of the present invention are preferably aromatic heterocyclic group having planar structure. Such aromatic

heterocyclic group has sufficient size for steric hindrance on the planar direction of molecule, and can generate electrostatic repulsion by different atoms, and also is expected to show stacking action by  $\pi$  electron of aromatic heterocyclic group.

Examples of such the aromatic heterocyclic group are, concretely, five membered or six membered aromatic heterocyclic group having one or two sulfur atom, oxygen atom or nitrogen atom. These aromatic heterocyclic groups can be condensed-ring, polycyclic or monocyclic group. Among them, monocyclic group is preferable due to steric size. These aromatic heterocyclic groups can have any substituents, but a group without having large substituent is preferable due to possibility to cause stereospecific limitation or generation of deteriorative hydrogen bonding. Examples of substituents are hydroxyl, amino, carbonyl, lower alkyl of carbon 1 -5, lower alkoxy, lower alkylamino or nitro.

Conventional chemical synthesis can be applied for methods of introducing groups to form steric hindrance, electrostatic repulsion or stacking action in the base.

Nucleic acid of the present invention is artificial nucleic acid which can be recognized by polymerase. Examples of polymerase can be any polymerase, preferably DNA polymerase and RNA polymerase. Recent studies on structural analysis of polymerase indicates that interaction of polymerase and nucleic acid is essentially identical with each other. Formation of base pair of the present invention relates to essential nature of polymerase, consequently, the base pair formation of the present invention can be utilized not only for DNA polymerase and RNA polymerase but also for all polymerase including reverse

transcriptase.

Further, configuration of nucleic acid can be calculated by analysis of molecular configuration or precise determination of distance between atoms. Consequently, by applying these results, chemical structure, which causes steric hindrance in one side and provides one or more hydrogen bonds, preferably two hydrogen bonds in other side, can be designed. Consequently, the present invention includes a method for designing artificial nucleic acid based on steric hindrance of the nucleic acid, preferably steric hindrance in the base part of nucleic acid, preferably in addition thereto, electrostatic repulsion and stacking action. In the designing base pair of the present invention, designing based on Watson-Crick type base pair is conventional, but Hoogsteen base pairing may also be applicable.

Nucleic acid of the present invention can be a nucleic acid designed by steric hindrance of nucleic acid, preferably designed by steric hindrance in the base part of the nucleic acid, and is preferable to form selective base pairing with each of artificial nucleic acid. Preferably, base pairing of artificial nucleic acid can be recognized by polymerase and more preferably the complementary strand can be constructed similar to the natural nucleic acid by and action of polymerase.

Nucleic acid of the present invention can be synthesized by conventional chemical synthesis but is not limited to that method. Chemical synthesis is exemplified in Fig. 3, Fig. 4 and Fig. 5.

Method for incorporating nucleic acid of the present invention into the nucleic acid sequence can be performed by applying conventional method for incorporation of natural nucleic acid or by applying similar method thereof.

For example, a method using DNA synthesizer, method for using polymerase and point mutation technology can be mentioned. Labeling can also be possible made as same as in the natural nucleic acid.

The present invention also includes nucleic acid which can be used for gene fragment or probe, and include nucleic acid molecule containing the nucleic acid of the present invention. The nucleic acid molecule of the present invention contains one or more nucleic acid of the present invention, and can be a single strand or double strands. Non-natural gene of the present invention includes natural gene in which whole of part of it is replaced by nucleic acid of the present invention, natural gene to which one or more nucleic acid of the present invention is added, or combination thereof. Such the non-natural gene of the present invention can be modified by the same or similar method used for the conventional modification of natural gene.

Consequently, nucleic acid molecule or non-natural gene of the present invention can be used for transformation of microorganisms by the same way as in the conventional natural gene by inserting suitable vector or phage and inserted into microorganisms to produce transformant containing the artificial nucleic acid of the present invention.

Further, new codon containing nucleic acid of the present invention can be designed. For example, the present novel artificial nucleic acid base is set as X and Y, combination thereof such as XXY, XYX, YXX, a combination by themselves, and AXA, TYT, CGX, ATX, which are combination of base of natural nucleic acid and artificial base of the present invention. Such codons can be designed. New codons can code natural amino acid, or non-natural amino acid. Further, functions such as transcription, transfer can be coded.



Accordingly, the present invention not only provide novel artificial nucleic acid, but also providing possibility of designing completely new genetic code by designing new codon containing nucleic acid of the present invention.

As a result of designing t-RNA corresponding to new codon of the present invention. New protein synthesis system can be designed by which large number of amino acid can be utilized.

Usable amino acid can be amino acid utilized on protein synthesis on liposome. Consequently, the present invention provides novel protein synthetic system using codon of the present invention.

Heretofore, some amino acids in the natural protein are very difficult to substitute non-natural amino acid, or insertion of non-natural amino acid into the natural protein is also very difficult. According to the protein synthesis system of the present invention, proteins containing desired non-natural amino acid can be produced by substituting or inserting the nucleic acid having codon of desired position into the nucleic acid of the present invention. And such the conversion of amino acid resulted to make screening functions of amino acid in the protein.

#### Brief Description of Drawings

Fig. 1 shows novel artificial nucleic acid base pair (X-Y) by utilizing steric hindrance of the present invention. R in Fig. 1 indicates 2-deoxy- $\beta$ -D-ribofuranosyl.

Fig. 2 shows novel artificial nucleic acid base pair (X2-Y) by utilizing base pairing caused by steric hindrance (Fig. 2 a and b) and steric hindrance, electrostatic repulsion and stacking action.

Fig. 3 shows synthetic scheme for amidite reagent for dX of nucleic acid having base X of the present invention.

Fig. 4 shows synthetic scheme of dYTP of nucleic acid having base Y of the present invention.

Fig. 5 shows synthetic scheme of amidite reagent of dx2 of nucleic acid having base X2 of the present invention.

Fig. 6 shows 20% polyacrylamide 7M urea gel electrophoresis of primer extension reaction by Klenow fragment using 5'-terminal  $^{32}\text{P}$  labeled primer 1 ( $0.5 \mu\text{M}$ ) and template 1, 3 ( $1 \mu\text{M}$ ) and various dNTP ( $150 \mu\text{M}$ ). Reaction was performed at  $17^\circ\text{C}$  for 30 minutes. B is a graph of the result.

Fig. 7 shows 20% polyacrylamide 7M urea gel electrophoresis of single nucleotide insertion reaction by Klenow fragment using 5'-terminal  $^{32}\text{P}$  labeled primer 2 ( $1 \mu\text{M}$ ) and template 1, 2, 3 ( $2 \mu\text{M}$ ) and various dNTP ( $150 \mu\text{M}$ ). Reaction was performed at  $17^\circ\text{C}$  for 30 minutes. A is electrophoretic pattern with 29% polyacrylamide 7M urea electrophoresis and B is a graph of the result.

Fig. 8 shows inhibitory experiment by dYTP on primer extension reaction by Klenow fragment using primer 2, template 1, 2, 3 and [ $\alpha$ - $^{32}\text{P}$ ]TTP or [ $\alpha$ - $^{32}\text{P}$ ]dCTP.

A: Primer extension reaction by Klenow fragment was performed at  $17^\circ\text{C}$  for 30 minutes. Primer 2 ( $1 \mu\text{M}$ ), template 3 ( $2 \mu\text{M}$ ) and [ $\alpha$ - $^{32}\text{P}$ ]TTP ( $150 \mu\text{M}$ ) are used.

dYTP, 0, 50, 150, 300 and  $500 \mu\text{M}$  are added.

Right lane 5: same experiment was performed by adding dATP ( $300 \mu\text{M}$ )

B: Primer extension reaction by Klenow fragment was performed at  $17^\circ\text{C}$  for

10 minutes. Primer 2 ( $1\mu\text{M}$ ), template 1 ( $2\mu\text{M}$ ) and [ $\alpha\text{-}^{32}\text{P}$ ]TTP ( $50\mu\text{M}$ ) are used.

dYTP, 0, 20, 100, 500 and  $1000\mu\text{M}$  are added.

Right lane 5: same experiment was performed by adding dATP ( $300\mu\text{M}$ )

C: Primer extension reaction by Klenow fragment was performed at  $17^\circ\text{C}$  for 30 minutes. Primer 2 ( $1\mu\text{M}$ ), template 2 ( $2\mu\text{M}$ ) and [ $\alpha\text{-}^{32}\text{P}$ ]TTP ( $50\mu\text{M}$ ) are used.

dYTP, 0, 20, 100, 500 and  $1000\mu\text{M}$  are added.

Right lane 5: same experiment was performed by adding dATP ( $300\mu\text{M}$ )

Fig. 9 shows primer extension reaction by Klenow fragment using 5'-terminal  $^{32}\text{P}$  labeled primer 3( $0.33\mu\text{M}$ ) and template 4, 5, 6, 7, 8, and 9 ( $1\mu\text{M}$ ) and various dNTP ( $150\mu\text{M}$ ). Reaction was performed at  $17^\circ\text{C}$  for 60 minutes.

Fig. 10 Electrophoresis of RNA generated by transcription of T7 RNA polymerase using various rNTPs and template 1-3 and [ $\alpha\text{-}^{32}\text{P}$ ]ATP.

Fig. 11: Transcription was performed similar to the case in Fig. 10 with rNTP and generated RNA was purified by electrophoresis and digested by RNase T2. Resulted product was analyzed by 2-dimension TLC.

Fig. 12: Incorporation of various base against X2 of the present invention. Drawing is replaced by photograph.

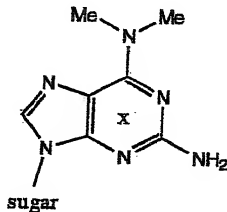
Fig. 13: Template containing X2 and rNTP were used. Generated RNA was purified by electrophoresis, then digested with RNase T2 and analyzed by 2-dimension TLC.

#### Best Mode for Carrying out the Invention

The present invention will be explained by the following examples in

detail.

One of artificial base, 2,6-diaminopurine, could form base pairing with thymine by hydrogen bonding at position-6 of thymine. In order not to form base pairing 2,6-diaminopurine with thymine, two bulky methyl groups were introduced into the amino group at position-6 of 2,6-diaminopurine for colliding with this group and keto group of thymine at position-6 by steric hindrance, and synthesized to design 2-amino-6-(N,N-dimethylamino) purine (hereinafter a base of which is designated as X) of the formulae:



Accordingly, X could not form base pairing with thymine an analogous base in which oxo group at position-6 of thymine is replaced by hydrogen, pyridine-2-one (hereinafter the base of which is designated as Y) could for base pairing with X (refer to Fig. 1). Lower part of Fig. 1 illustrates binding not to form base pairing these bases X and Y with other bases.

In order to examine formation of selective novel nucleic acid base pair by utilizing steric hindrance in the base pair X-Y, primer extension method of DNA and transcriptional reaction synthesizing RNA from DNA were applied. The primer extension method includes annealing template DNA oligomer with

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a primer oligomer, and adding DNA polymerase and 2'-deoxynucleotide-5'-triphosphate (dNTP) to extend complementary sequence of the template at 3'-terminal of the primer. Klenow fragment, which is deleted 5'-exonuclease from DNA polymerase I, one of DNA polymerase originated from *E. coli*, and T7 RNA polymerase, RNA polymerase originated from T7 phage, were herein used. Both enzymes are commonly used at present.

In order to incorporate X into the template DNA, amidite reagent of dX was synthesized. Also template DNA containing dX having base sequences hereinbelow (Template 3, 5, 6, 7, 8 and 9) and template DNA (Template 1, 2 and 4) and their primer (Primer 1, 2 and 3) for use of control experiments were synthesized.

Template DNA containing dX (Template 3, 5, 6, 7, 8 and 9):

Template 3: dtgctctxtcttccctccctatagtgagtcgtattat  
Template 5: dagctxtgtgtgtctccggtacaactaggc  
Template 6: dagctxxgtgtgtctccggtacaactaggc  
Template 7: dagctxixgtgtgtctccggtacaactaggc  
Template 8: dagctxtgxtgtgtctccggtacaactaggc  
Template 9: dagctxtgtxtgtgtctccggtacaactaggc

Template for using control experiment (Template 1, 2 and 4)

Template 1: dtgctctatcttccctccctatagtgagtcgtattat  
Template 2: dtgctctgtcttccctccctatagtgagtcgtattat  
Template 4: dagctgtgtgtgtgtctccggtacaactaggc

Primer (Primer 1, 2 and 3)

Primer (Primer 1, 2 and 3)

Primer 1 : dgcactcactataggg

Primer 2 : dctatagggaggaga

Primer 3 : dgcctagttgtaccg

Substrates, dYTP and rYTP, were also synthesized (refer to Fig. 4)

5'-terminal of the primer was labeled with  $^{32}\text{P}$  using T4 polynucleotide kinase and [ $\alpha$ - $^{32}\text{P}$ ] ATP. Primer labeled with  $^{32}\text{P}$  ( $0.5 \mu\text{M}$ ) and template 1 and 3 ( $1 \mu\text{M}$ ) and various dNTP ( $150 \mu\text{M}$ ), wherein N means base, were used for primer extension by Klenow fragment ( $0.2 \text{ unit}/\mu\text{l}$ ) at  $17^\circ\text{C}$  for 30 minutes.

A combination of primer and template used in the experiment is shown hereinbelow.

A case using template 1:

Primer 1 : 5' -  $^{32}\text{P}$ CGACTCACTATAGGG

Template 1: 3' -TATTATGCTGAGTGATATCCCTCCTTCTATCTCGT

A case using template 3:

Primer 1 : 5' -  $^{32}\text{P}$ CGACTCACTATAGGG

Template 3: 3' -TATTATGCTGAGTGATATCCCTCCTTCTATCTCGT

The thus obtained product was electrophoresed using 20% polyacrylamide 7M urea gel and analyzed by using imaging plate (Phosphoroimager analysis). Result is shown in Fig. 6. In Fig. 6, left five, i.e. AG, AGC, AGT, AGY and AGCT, indicates cases using template 1, and right five indicates cases using template 3. Results indicate that a base y was incorporated into complementary strands of A, G and X. To the complementary

strand X was incorporated also C and T in addition to Y (refer to Fig. 6).

For quantitative analysis of the incorporation, same experiments were conducted by adding only dNTP (159  $\mu$ M) using primer 2 (1  $\mu$ M) labeled with p32 at 5'-terminal and template 1, 2 and 3 (2  $\mu$ M).

Primer and template used in the experiments are shown below.

A case using template 1:

Primer 2 : 5'-<sup>32</sup>pCTATAGGGAGGAGA

Template 1: 3'-TATTATGCTGAGTGATATCCCTCCTTCTATCTCGT

A case using template 2:

Primer 2 : 5'-<sup>32</sup>pCTATAGGGAGGAGA

Template 2: 3'-TATTATGCTGAGTGATATCCCTCCTTCTGTCTCGT

A case using template 3:

Primer 2 : 5'-<sup>32</sup>pCTATAGGGAGGAGA

Template 3: 3'-TATTATGCTGAGTGATATCCCTCCTTCTXTCTCGT

Results are shown in Fig. 7 A and B. As a result, Y was incorporated into complementary strands of A, G and X at 78%, 48% and 42%, respectively, and Y, C and T were incorporated into complementary strand of X, at 41%, 9.5% and 13%, respectively. (Refer to Fig. 7)

Since Y was incorporated independently into not only X but also A and G, the following experiments were conducted in order to find out to what strands Y was incorporated when T and C were coexisted.

Primer 2 without labeling and template 2 were annealed, and [ $\alpha$ -<sup>32</sup>P]

TTP and various amounts of dYTP were added thereto to find out ratio of inhibition of incorporation of [ $\alpha$ - $^{32}$ P] TTP into X by dYTP was investigated. Simultaneously with addition of dATP, effect of the said inhibition on incorporation of A into complementary strand of T next to X was investigated (refer to Fig. 8 A). As a result, when dYTP was added almost equivalent level of [ $\alpha$ - $^{32}$ P] TTP, incorporation of [ $\alpha$ - $^{32}$ P] TTP into X was inhibited at 50%. Same experiments were conducted by using template 1 and 2 for A and G. dYTP did not inhibit incorporation of [ $\alpha$ - $^{32}$ P] TTP into the complementary strand of A and the incorporation of [ $\alpha$ - $^{32}$ P] CTP into the complementary strand of G. (Refer to Fig. 8 B for template 1 and C for template 2). Consequently, incorporation of dYTP into A and G was suppressed by coexisting TTP and dCTP.

In order to search effect of incorporation of Y, C and T into the complementary strand of X, when two X are presented on the template,  $^{32}$ P labeled primer 3 at 5'-terminal and template 4, 5, 6, 7, 8 and 9 were used for primer extension method. As a result, when two X were continued on the template, polymerase reaction was terminated at the position where two continuous X were existed whenever using any bases. In only the case where another base was incorporated between two X, only Y was incorporated into the complementary strand of the second X in the two X and continued synthesis of complementary strand (refer to Fig. 9).

Similarly, a transcription reaction by RNA polymerase using DNA containing X as template was examined. Using template 1-3, promoter region on this strand was duplicated, and the transcription reaction was examined using T7 RNA polymerase by adding [ $\alpha$ - $^{32}$ P] ATP.



A combination of primer region and template used in this experiment is shown as follows.

A case using template 1-3

Coding strand : 5'-ATAATACGACTCACTATAGGG

Template 1-3 : 3'-TATTATGCTGAGTGATATCCCTCCTTCTNTCTCGT

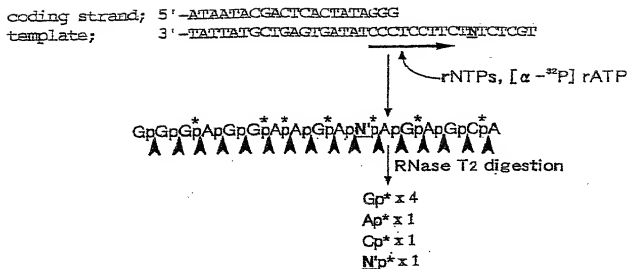
(template 1: N = A

template 2: N = G

template 3: N = X)

Result is shown in Fig. 10. In case of using template 3 (N = X), a band corresponding to a product as a result of selective incorporation of Y to X is observed. Trace amount of U was incorporated. In the case of template 1 (N = A), not only U but also Y was found to incorporated into the complementary strand of A. In the case of the template 2 (N = G), only C was incorporated, and almost no production as a result of incorporation of Y was observed.

Transcription reaction in the presence of rNTP was conducted. Using the template 1 (N = A) and the template 3 (N = X), the same as in the previous experiments, the transcription reaction was examined using T7 RNA polymerase by adding [ $\alpha$ -<sup>32</sup>P] ATP under the condition of rATP 2 mM, rGTP 2 mM, rCTP 2 mM, UTP 2 mM, and rYTP 1 mM, then generated full length of RNA was digested completely by RNase T2, nucleotide labeled at 3'-terminal was analyzed by 2-dimension TLC. Outline of this experiment is shown as follows.



Results are shown in Fig. 11. In Fig. 11, spots encircled are indicated as spots observed by UV. In Fig. 11, A indicates the case of template 3 ( $N = X$ ) and B in Fig. 11 indicates the case of template 1 ( $N = A$ ). Theoretical values and experimental values in each case are shown in Table 1 as follows.

Table 1, Theoretical and measured values of the base for each template

Base	Template 3 ( $N=X$ )		Template 1 ( $N=A$ )	
	Theoretical Value	Measured Value	Theoretical Value	Measured Value
Gp	4	4	4	4
Ap	1	1.05	1	0.92
Cp	1	0.94	1	0.78
Up	0	0.08	1	0.98
Yp	1	0.82	0	0.04

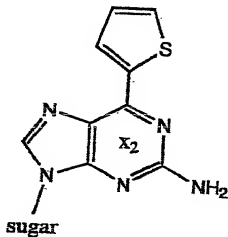
Result indicates that Y is almost selectively incorporated in case of transcription reaction using the template 3 ( $N = X$ ) and trace amount of U is detected. In the case of template 1 ( $N = A$ ), no incorporation of Y is observed.

As explained hereinabove, the base X designed as such does not form base pairing with thymine, but the base such as analogous pyridine-2-one (base X) in which oxo at position-6 of thymine is replaced by hydrogen is able to form base pair with X (refer to Fig. 2 a and b). Consequently, formation of selective nucleic base pair of X-Y has detected.

Although formation of base pair of natural base thymine (or uridine) (refer to Fig. 2 b) could be excluded, simultaneously low rate of incorporation of dYTP by Klenow fragment was observed due to disadvantageous effect on stacking between bases as well as insufficient suppression of incorporation of thymidine triphosphate (dTTP) to dx.

Consequently, attempts were performed to incorporate aromatic substituents, which have no deteriorating effect on stacking between bases, to position-6 in dx as the replacement of dimethylamino group.

In the experiments, an example of incorporation of thiophene at position-6 is illustrated as follows.



2-amino-6-(2-thienyl)-9-(2-deoxy- $\beta$ -D-ribofuranosyl) purine [dx 2: the new base of which is designated as X2, and the previously synthesized 2-amino-6-(N,N-dimethylamino)-9-(2-deoxy- $\beta$ -D-ribofuranosyl) purine is designated as dx] was synthesized. Incorporation of dYTP and rYTP for templates including this base was examined.

Outline of preparation of amidite reagent for dx for synthesis of template DNA is illustrated in Fig. 5. In detail, refer to example 2. This amidite reagent could show similar coupling rate as same as of the commercially available amidite reagent.

Using Klenow fragment (exo<sup>-</sup>), incorporation of dYTP on dx2 in the template (refer to example 10).

Following base sequences were used as template and primer.

Primer 5' -<sup>32</sup>PACTCACTATAGGGAGGAACA-

Template 3' -TATTATGCTGAGTGATATCCCTCCTTCT-N-TCTCGT

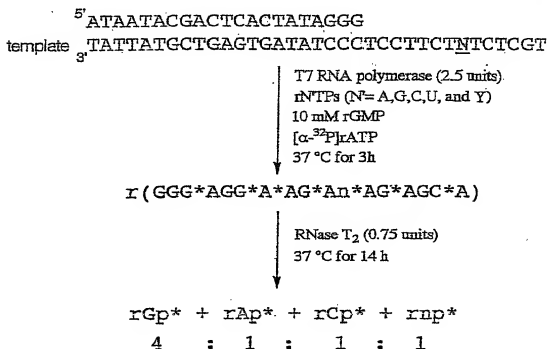
In the template, a position indicated by N was bound with base X or base X2 (for experimental) or base A (for control), and incorporation experiments of various bases were conducted. Results are shown in Fig. 12, in which lanes 1 and 2 indicate control experiments for incorporation of cytosine (C) and thymine (T) on adenine (A).

Results indicate that rate of incorporation of dYTP on dx is 21% (lane 3), and that on dx2 is increased up to 40% (lane 8). As compared with the incorporation rate of 57% in the case of dTTP on natural type dA under the same condition, some improvement of incorporation was observed by using dx2. Although comparing with the case of dx, incorporation of dCTP is increased by using dx2 (22%) (lane 11), it is not so improved value as compared with the

incorporation of dYTP (40%).

As a result of experiment using Klenow fragment (exo<sup>+</sup>), incorporation rate of dCTP on dx2 was increased by using dx2 in place of dx. This might be due to interaction of 4-amino group of cytosine and sulfur atom in thiophene in dx2 (refer to Fig. 2 f). As is the case that sulfur atom in thiophene of dx2 is directed to a plane of base pair, electrostatic repulsion against 4-keto group of thymine (T) will be expected. This indicates that electrostatic repulsive force (refer to Fig. 2 e) can be used in addition to steric hindrance as a factor for hinder formation of base pairing. Accordingly, in thiophene in dx2, sulfur atom side might be directed to the plane of base pair.

Incorporation of rYTP into RNA on dx2 in the template by T7 RNA polymerase was examined according to the reaction (example 11):



RNA having a sequence of the formula:



wherein n is a base corresponding to a base N, asterisk of the right shoulder means labeling, is digested by RNase T2, then ratio of each nucleotide was calculated by 2-diemntion TLC (cellulose resin).

In Fig. 13, development of TLC is shown. Ratio of composition of each nucleotide is shown in Table 2 hereinbelow.

Table 2

Template	rGp <sup>*</sup>	rAp <sup>*</sup>	rCp <sup>*</sup>	rUp <sup>*</sup>	rYp <sup>*</sup>
N = x <sub>2</sub>	3.982(4)	1.052(1)	0.950(1)	0.047(0)	0.969(1)
N = A	3.939(4)	1.035(1)	0.995(1)	1.032(1)	not detected(0)

A parenthesis in the table indicates theoretical value.

Result indicates that using dx2, rYTP can be incorporated against dx2 with high selectivity. Good result has been obtained in case of using dx as a template in the previous experiment, and in case of using dx2 as a template to perform transcription in the similar condition, result of analysis on nucleotide incorporated in RNA on dx2 indicated that the similar high selectivity of incorporation of rYTP on dx2 was obtained.

As explained hereinabove, the present invention provides selective formation of base pair which has never achieved in the heretofore reported artificial base pair. Further, the present invention demonstrates that such the selective formation of base pair can be achieved by steric hindrance of base

pair, preferably by applying steric hindrance and electrostatic repulsion as well as stacking action. Bases used in the above experiments are shown as illustration only of the present invention. This fact proves correctness of the idea of the present invention for achieving selective formation of artificial nucleic acid base pair. Consequently, the present invention is never limited within the bases concrete illustrated hereinbefore, and all base pairs generated according to the idea of the present invention are within the scope of the present invention.

Further, the fact that base pairs of the present invention could be recognized by natural synthetases has actually proved, and the base pairs of the present invention could also be used in synthetic and transcriptional systems of natural DNA and RNA as similar to the natural base pairs. Consequently, the present invention provides concept for formation of novel artificial base pair which can be applied and achieved in the systems on functional expression of natural gene. Such the artificial nucleic acid base pair of the present invention can be applied not only on the protein synthesis system or functional nucleic acid but also on the solution of functions and elucidation of natural gene systems.

### Examples

Following examples illustrate the present invention but are not construed as limiting the present invention.

#### Example 1:

Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-[5'-O-

dimethoxytrityl-3'-O-[[[(diisopropylamino)-2-cyanoethoxy] phosphino]-2'-deoxy- $\beta$ -D-ribofuranosyl] purine (10) (refer to Fig. 3)

(A) Synthesis of 2-amino-6-(N,N-dimethylamino)-9-(2',3,5'-tri-O-acetyl- $\beta$ -D-ribofuranosyl] purine (2):

2-amino-6-chloro-9-(2',3',5'-tri-O-acetyl- $\beta$ -D-ribofuranosyl) purine (1) [M. J. Robins and B. Uznanski, Can. J. Chem., 59, 2601-2607 (1981)](18.6 mmol, 7.96 g) was dehydrated three times azeotropically with anhydrous pyridine, and dissolved in anhydrous pyridine, then dimethylamine hydrochloride (55.8 mmol, 4.55 g) and diisopropylethylamine (74.4 mmol) were added thereto with stirring at room temperature. The mixture was stirred at room temperature for 15 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue, and the organic layer was washed 3 times with water, 2 times with 5% aqueous sodium hydrogen carbonate, once with water and 2 times with 10% aqueous citrate solution, then the organic layer was dried with magnesium sulfate, and dried in vacuo after filtration. The residue was treated with azeotropic distillation with toluene until no odor of pyridine was noted, the product was purified by silica-gel chromatography (dichloromethane-ethanol) to obtain the product (2) 5.42 g (12.4 mmol)(67%).

$^1\text{H-NMR}$  (500.13 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.56 (s, 1H, H8), 6.02 (d, 1H, H1', J = 5.0 Hz), 5.95 (dd, 1H, H2', J = 5.0 Hz), 5.79 (t, 1H, H3', J = 5.0 Hz), 4.69 (s, 2H, 2-NH<sub>2</sub>), 4.42-4.45 (m, 1H, H4'), 4.34-4.40 (m, 2H, H5', H5''), 3.43 (br, 6H, N-CH<sub>3</sub>), 2.13, 2.10, 2.08 (s, 3H, Ac).



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(B) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-[2',3',5'-tri-O-acetyl- $\beta$ -D-ribofuranosyl] purine (3)

A compound (2) obtained in the above (A) (10 mmol, 4.36 g) was azeotropically dehydrated three times and dissolved in anhydrous pyridine (180 ml). Under stirring at room temperature, benzoyl chloride (15 mmol, 1.74 ml) was added and the mixture was stirred at room temperature for 14 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue, and the organic layer was washed 2 times with 5% aqueous sodium hydrogen carbonate and once with water. The organic layer was dried with magnesium sulfate and filtered, then concentrated in vacuo. The residue was treated by azeotropic distillation until the residue showed no odor of pyridine. The residue was purified by silica-gel column chromatography (hexane-dichloroethane) to obtain the product (3) 3.53 g (6.53 mmol) (65%).

$^1\text{H-NMR}$  (500.13 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.46 (s, 1H, H8), 7.96 (d, 2H, Bz-m,  $J = 10.0$  Hz), 7.75 (s, 1H, NHBz), 7.55 (dd, 1H, Bz-p,  $J = 7.5$  Hz), 7.48 (t, 2H, H Bz-o,  $J = 7.5$ ), 6.08 (d, 1H, H1',  $J = 3.0$  Hz), 5.96-6.01 (m, 2H, H2', H3'), 4.39-4.50 (m, 3H, H4', H5', H5''), 3.48 (br, 6H,  $\text{N-CH}_3$ ), 2.15 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 3H, Ac).

(C) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-( $\beta$ -D-ribofuranosyl) purine (4)

To a compound (3) obtained hereinabove (6.53 mmol, 3.53 g), pyridine-methanol-water (65 : 30 : 5) 50 ml was added and stirred in the ice-water bath. 2M sodium hydroxide-pyridine-methanol-water (65 : 30 : 5) 50 ml

was added and stirred for 15 minutes in the ice-water bath. After confirming completion of the reaction, ammonium chloride (5.21 g) was added to the reaction mixture and concentrated in vacuo until volume reached up to 40 ml. Chloroform was added to the solution, and the organic layer was extracted. Then the aqueous layer was extracted twice with chloroform-pyridine. The organic layer was collected and dried with magnesium sulfate. The filtrate was concentrated up to volume of 10 ml in vacuo. Toluene was added thereto and concentrated in vacuo to precipitate crystals. Crystals were collected by filtration and dried in vacuo at 90°C to obtain the product (4) 2.87 g.

<sup>1</sup>H-NMR (500.13 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 8.28 (s, 1H, H8), 7.92 (dd, 2H, Bz-m, J = 7.0 Hz), 7.57 (dd, 1H, Bz-p, J = 7.3 Hz), 7.49 (t, 2H, H Bz-o, J = 7.5), 5.91 (d, 1H, H1', J = 4.0 Hz), 5.48 (d, 1H, OH, J = 5.5 Hz), 5.15 (d, 1H, OH, J = 4.0 Hz), 5.04 (t, 1H, OH, J = 1.0 Hz), 4.56 (t, 1H, H2', J = 10.0 Hz), 4.17 (d, 1H, H3', J = 3.0 Hz), 3.93 (d, 3H, H4', J = 3.5 Hz), 3.63-3.65 (m, 1H, H5'), 3.52-3.56 (m, 1H, H5''), 3.48 (br, 6H, N-CH<sub>3</sub>).

(D) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(3',5'-O-tetraisopropylidisiloxanyl- $\beta$ -D-ribofuranosyl) purine (5)

A compound (4) obtained in the above (4) (5.0 mmol, 2.07 g) was dehydrated three times azeotropically with anhydrous pyridine and dissolved in anhydrous pyridine (50 ml), then 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane (5.5 mmol, 1.76 ml) was added and stirred at room temperature for 14 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue. The organic layer was washed twice with

5% aqueous sodium hydrogen carbonate and once with saturated sodium chloride solution. The organic layer was dried with magnesium sulfate, filtered and dried in vacuo. Azeotropic treatment was repeated until no odor of pyridine in the residue was noted. Then the residue was purified by silica-gel chromatography (dichloromethane-methanol) to obtain the product (5) 2.63 g (4.0 mmol)(80%).

<sup>1</sup>H-NMR (500.13 MHz, CDCl<sub>3</sub>) δ : 8.22 (s, 1H, H8), 7.89 (d, 2H, Bz-m, J = 5.0 Hz), 7.80 (s, 1H, NHBz), 7.55 (dd, 1H, Bz-p, J = 7.5 Hz), 7.48 (t, 2H, Bz-o, J = 7.5 Hz), 5.91 (s, 1H, H1'), 4.84 (dd, 1H, H3', J = 5.5 Hz), 4.50 (d, 1H, H2', J = 5.5 Hz), 4.07-4.20 (m, 2H, H4', H5'), 4.06 (d, 1H, H5'', J = 13.0 Hz), 3.48 (br, 6H, N-CH<sub>3</sub>), 0.95-1.08 (m, 28H, iPr).

(E)      Synthesis      of      2-benzamino-6-(N,N-dimethylamino)-9-(2'-O-phenoxythiocarbonyl-3',5'-O-tetraisopropylidisiloxanyl- β -D-ribofuranosyl) purine (6)

The compound (5) obtained in the above (D) was dehydrated azeotropically three times with anhydrous toluene and dissolved in anhydrous dichloromethane (40 ml). 1-methylimidazole (7.96 mmol, 0.64 ml) and chlorothio carbonate phenyl (5.57 mmol, 0.77 ml) were added with stirring at room temperature, then stirred at room temperature for 16 hours. After confirming completion of the reaction by TLC, 5% aqueous sodium hydrogen carbonate was added to the reaction mixture. After extracted the organic layer, the organic layer was washed one with aqueous 5% sodium hydrogen carbonate, once with water, twice with aqueous 10% citrate solution and once with water, in this order, the organic layer was dried with magnesium sulfate, filtered and

dried in vacuo. The residue was purified by silica-gel column chromatography (dichloromethane-methanol) to obtain the product (6) 2.96 g (3.73 mmol) (94%).

<sup>1</sup>H-NMR (500.13 MHz, DMSO-d<sub>6</sub>) δ: 8.17 (s, 1H, H8), 7.87 (d, 2H, Bz-m, J = 3.0 Hz), 7.79 (s, 1H, NHBz), 7.55 (t, 1H, Bz-p, J = 7.5 Hz), 7.47 (t, 2H, H Bz-o, J = 7.5 Hz), 7.41 (d, 2H, PhO-o, J = 7.5 Hz), 7.29 (t, 2H, PhO-m, J = 7.5 Hz), 7.13 (d, 1H, PhO-p, J = 10.0 Hz), 6.39 (d, 1H, H2', J = 5.0 Hz), 6.11 (s, 1H, H1'), 5.14-5.17 (m, 1H, H3'), 4.23-4.26 (m, 1H, H5'), 4.07-4.12 (m, 1H, H4', H5"), 3.48 (br, 6H, N-CH<sub>3</sub>), 0.99-1.15 (m, 28H, iPr).

(F) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(2'-deoxy-3',5'-O-tetraisopropylidisiloxanyl-β-D-ribofuranosyl) purine (7)

The compound (6) obtained in the above (E) was dehydrated azeotropically three times with anhydrous toluene, and dissolved in anhydrous toluene (88 ml). 2,2'-azo-bis-isobutyronitrile (0.746 mmol, 122 mg) was added thereto with stirring at room temperature and added argon gas with bubbling for 1 hours at room temperature. Thereto was added tributyltin hydride (5.60 mmol, 1.51 ml) and stirred at 75°C for 3.5 hours. After confirming completion of the reaction by TLC, the reaction mixture was concentrated in vacuo. The residue was purified by silica-gel column chromatography (dichloromethane-methanol) to obtain the product (7) 2.27 g (3.55 mmol) (95%).

<sup>1</sup>H-NMR (500.13 MHz, CDCl<sub>3</sub>) δ: 8.24 (s, 1H, H8), 7.90 (d, 2H, Bz-m, J = 5.0 Hz), 7.83 (s, 1H, NHBz), 7.54 (t, 1H, Bz-p, J = 7.5 Hz), 7.48 (t, 2H, H Bz-o, J = 7.5 Hz), 6.29 (dd, 1H, H1', J = 7.5 Hz), 4.80-4.83 (m, 1H, H3'), 3.97-4.07 (m, 2H, H5', H5"), 3.86-3.88 (m, 1H, H4'), 3.50 (br, 6H, N-CH<sub>3</sub>), 2.68-2.71 (m, 1H, H2'), 2.59-2.63 (m, 1H, H2"), 1.03-1.09 (m, 28H, iPr).

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(G) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(2'-deoxy- $\beta$ -D-ribofuranosyl) purine (8)

The compound (7) obtained in the above (F) (3.55 mmol, 2.27 g) was added to 1M solution of tetrabutylammonium fluoride - tetrahydrofuran (14 ml) and stirred at room temperature for 15 minutes. After confirming completion of the reaction by TLC, the reaction mixture was concentrated in vacuo. The residue was dissolved in chloroform, washed with small amount of water, and the aqueous layer was extracted 4 times with chloroform. The organic layer was dried with magnesium sulfate. The filtrate was concentrated in vacuo and the residue was treated azeotropically with toluene until no odor of pyridine was noted. The residue was dissolved in methanol. Dichloromethane was added dropwise as little as possible to crystallize the product. The crystals were collected by filtration and dried in vacuo to obtain the product (8) 0.964 g (2.42 mmol) (68%).

$^1\text{H-NMR}$  (500.13 MHz,  $\text{DMSO-d}_6$ )  $\delta$ : 8.23 (s, 1H, H8), 7.84 (d, 2H, Bz-m,  $J = 7.5$  Hz), 7.50 (t, 1H, Bz-p,  $J = 7.3$  Hz), 7.42 (t, 2H, H Bz-o,  $J = 7.5$  Hz), 6.25 (t, 1H, H1',  $J = 7.0$  Hz), 5.21 (s, 1H, OH), 4.89 (s, 1H, OH), 4.33 (s, 1H, H3'), 3.77 (s, 1H, H4'), 3.50-3.53 (m, 1H, H5'), 3.43-3.46 (m, 1H, H5''), 3.48 (br, 6H,  $\text{N-CH}_3$ ), 2.56-2.61 (m, 1H, H2'), 2.16-2.18 (m, 1H, H2'').

(H) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-(5'-O-dimethoxytrityl-2'-deoxy- $\beta$ -D-ribofuranosyl) purine (9)

The compound (8) (1.47 mmol, 0.585 g) was azeotropically dehydrated three times with anhydrous pyridine and dissolved in anhydride pyridine (10

ml). 4,4'-dimethoxy tritylchloride (1.61 mmol, 547 mg) was added with stirring at room temperature, then stirred at room temperature for 1.5 hours. After confirming completion of the reaction by TLC, water was added to the reaction mixture and concentrated in vacuo. Chloroform was added to the residue and the organic layer was washed twice with aqueous 5% sodium hydrogen carbonate and once with water, then the organic layer was dried with magnesium sulfate, filtered and concentrated in vacuo. The residue was azeotropically distilled with toluene until no odor of pyridine was noted, and was purified by silica-gel column chromatography (dichloromethane - methanol - 0.5% triethylamine) to obtain the product (9) 0.99 g (1.41 mmol) (96%).

$^1\text{H-NMR}$  (270.16 MHz,  $\text{CDCl}_3$ )  $\delta$ : 8.20 (s, 1H, H8), 7.79 (s, 1H, NHBz), 7.77 (d, 2H, Bz-m,  $J = 1.4$  Hz), 7.76 (d, 1H, Bz-p,  $J = 3.5$  Hz), 7.14-7.51 (m, 11H, H Bz-o, DMTrI, 6.72 (dd, 4H, DMTr), 6.45 (t, 1H, H1',  $J = 6.5$  Hz), 4.78 (m, 1H, H3'), 4.14 (m, 1H, H4'), 3.74 (s, 6H,  $\text{OCH}_3$ ), 3.50 (br, 6H,  $\text{N-CH}_3$ ), 3.39-3.47 (m, 1H, -H5'), 3.30-3.33 (m, 1H, H5''), 2.80-2.85 (m, 2H, H2', H2'').

(I) Synthesis of 2-benzamino-6-(N,N-dimethylamino)-9-[5'-O-dimethoxytrityl-3'-O-[[[(diisopropylamino)-2-cyanoethoxy] phosphino]-2'-deoxy- $\beta$ -D-ribofuranosyl] purine (10)

The compound (9) (0.864 mmol, 0.605 g) was azeotropically distilled three times with anhydrous pyridine and twice with anhydrous tetrahydrofuran and dissolved in anhydrous tetrahydrofuran (6 ml). N,N-diisopropylethylamine (2.59 mmol, 0.452 ml) and chloro-2-cyanoethoxy-N,N-diisopropyl-aminophosphine (1.73 mmol, 0.385 ml) were added with stirring at

room temperature and further stirred at room temperature for 2 hours. After confirming completion of the reaction by TLC, anhydrous methanol was added to the reaction mixture to terminate the reaction. Ethyl acetate was added to the reaction mixture, and the organic layer was washed once with 5% aqueous sodium hydrogen carbonate and three times with saturated aqueous sodium chloride solution. The organic layer was dried with anhydrous sodium sulfate and concentrated in vacuo after filtration. The residue was purified by silica-gel column chromatography (dichloromethane -methanol - 2% triethylamine), and dissolved in small volume of chloroform, and reprecipitated by adding hexane to obtain the product (10) 0.574 g (0.638 mmol) (74%).

<sup>1</sup>H-NMR (270.16 MHz, CDCl<sub>3</sub>) δ : 8.14 (s, 1H, H8), 8.13 (s, 1H, H8), 7.72 (s, 1H, NHBz), 7.65-7.70 (m, 2H, Bz-m), 7.16-7.47 (m, 12H, H Bz-p,o, DMTr), 6.70-6.75 (m, 4H, DMTr), 6.27-6.41 (m, 1H, H1'), 4.63-4.80 (m, 1H, H3'), 4.20-4.27 (m, 1H, H4'), 3.74 (s, 6H, OCH<sub>3</sub>), 3.24-3.72 (m, 10H, H5', H5'', NCH(CH<sub>3</sub>)<sub>2</sub>, N-CH<sub>3</sub>), 2.83-3.00 (m, 1H, H2'), 2.40-2.64 (m, 5H, H2'', OCH<sub>2</sub>CH<sub>2</sub>CN), 1.06-1.19 (m, 12H, NCH(CH<sub>3</sub>)<sub>2</sub>).

<sup>31</sup>P-NMR (109.36 MHz, CDCl<sub>3</sub>) δ : 149.25.

#### Example 2:

Synthesis of 2-isobutyrylamino-6-(2-thienyl)-9-[2-deoxy-3-O-[diisopropylamino]-(2-cyanoethoxy)] phosphino-5-O-dimethoxytrityl- β -D-ribofuranosyl] purine (22) (Synthetic route is shown in Fig. 5)

(A) Synthesis of 2-isobutyrylamino-6-iodo-9-(2-deoxy-3,5-di-O-isobutyryl- β -D-ribofuranosyl] purine (18)

2-isobutyrylamino-6-amino-9-(2-deoxy-3,5-di-O-isobutyryl- $\beta$ -D-ribofuranosyl] purine (17) [Babara L. Gaffney, Luis A. Marky and Roger A. Jones, Tetrahedron, 40, 3-13 (1984)] 2.38 g (5 mmol) was heated at 60°C under argon atmosphere. n-pentyl nitrite 13.5 ml (0.10 mol) and diiodomethane 25 ml (0.31 mol) were rapidly added and suspended. The mixture was irradiated by visible light using 200 W halogen tungsten lamp at the distance from light source 2 cm for 3 hours under well stirring at 60°C. To the reaction mixture, saturated aqueous sodium sulfite 30 ml was added and stirred at room temperature for 3 hours. Thereafter, saturated aqueous sodium sulfite 120 ml and chloroform 150 ml were added to separate the layers. The aqueous layer was extracted twice with chloroform. The thus obtained organic layer was dried with anhydrous magnesium sulfate and concentrated. The residue was purified by short column (developer: ethyl acetate : dichloromethane = 1 : 4) to obtain the product (18) 1.01 g (1.72 mmol) (34.4%).

<sup>1</sup>H-NMR (270 MHz, CDCl<sub>3</sub>)  $\delta$  :

8.19 (s, 1H), 8.14 (bs, 1H), 6.42 (dd, J = 7.4, 6.4 Hz, 1H), 5.44 (m, 1H), 4.41 (m, 2H), 4.34 (m, 1H), 3.00 (m, 1H), 2.80 (m, 1H), 2.58 (m, 3H), 1.17 (m, 18H).

(B) Synthesis of 2-isobutyrylamino-6-(2-thienyl)-9-(2-deoxy-3,5-di-O-isobutyryl- $\beta$ -D-ribofuranosyl] purine (19)

The compound (18) 294 mg (0.5 mmol) obtained in the above (A) was dissolved in thiophene 80 ml under argon atmosphere and the solution was transferred into the photochemical reaction vessel (Pyrex). Ultraviolet ray was irradiated using 400 W mercury lamp for 24 hours under argon atmosphere. The reaction mixture after irradiation was concentrated, and the residue was



purified using short column (developer: isopropanol : dichloromethane = 3 : 197) to obtain the product (19) 212 mg (0.39 mmol) (78.0%).

<sup>1</sup>H-NMR (270 MHz, CDCl<sub>3</sub>) δ :

8.63 (dd, J = 3.8, 1.2Hz, 1H), 8.17 (s, 1H), 8.10 (bs, 1H), 7.64 (m, 1H), 7.25 (m, 1H), 6.47 (dd, J = 7.9, 1.8 Hz, 1H), 5.44 (m, 1H), 4.43 (m, 2H), 4.37 (m, 1H), 3.18 (m, 1H), 3.00 (m, 1H), 2.61 (m, 3H), 1.24 (m, 18H).

(C) Synthesis of 2-isobutyrylamino-6-(2-thienyl)-9-(2-deoxy- β -D-ribofuranosyl) purine (20)

The compound (19) 212 mg (0.39 mmol) obtained by the above (B) was dissolved in 1 M sodium hydroxide solution (pyridine - methanol - water = 13 : 6 : 1) 1.95 ml under ice-cooling and stirred for 15 minutes. The reaction mixture was neutralized by adding aqueous 5% ammonium chloride. Further added 1.2 g Celite to the mixture and the solvent was removed completely under reduced pressure. The residue was purified by short column (developer: 5 - 7% ethanol - dichloromethane) to obtain the product (20) 147 mg (0.37 mmol) (93.6%).

<sup>1</sup>H-NMR (270 MHz, DMSO-d<sub>6</sub>) δ :

10.45 (bs, 1H), 8.69 (s, 1H), 8.60 (d, J = 3.5 Hz, 1H), 7.90 (d, J = 4.6 Hz, 1H), 7.32 (dd, J = 4.6, 3.5 Hz, 1H), 6.39 (t, J = 6.6 Hz, 1H), 5.34 (d, J = 3.8 Hz, 1H), 4.91 (t, J = 5.3 Hz, 1H), 4.44 (m, 1H), 3.55 (m, 2H), 2.96 (m, 1H), 2.74 (m, 1H), 2.33 (m, 1H), 1.11 (m, 6H).

(D) Synthesis of 2-isobutyrylamino-6-(2-thienyl)-9-(2-deoxy-5-O-dimethoxytrityl-β -D- ribofuranosyl) purine (21)

The compound (20) 98 mg (0.24 mmol) obtained in the above (C) was azeotropically distilled three times with anhydrous pyridine. The residue was dissolved in anhydrous pyridine 2 ml, added triethylamine 35 ml, dimethylaminopyridine 1.4 mg and dimethoxytrityl chloride 85 mg were added thereto and stirred at room temperature for overnight. Ethyl acetate 25 ml was added to the reaction mixture. The mixture was treated with water for three time for separation to obtain organic layer. Each aqueous layer was washed with ethyl acetate. The organic layer was collected, dried with anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by using short column (developer: 25 - 50% ethyl acetate - dichloromethane) to obtain the product (21) 132 mg (0.19 mmol) (76.7%).

$^1\text{H-NMR}$  (270 MHz,  $\text{CDCl}_3$ )  $\delta$  :

8.64 (dd,  $J = 3.6, 0.9$  Hz, 1H), 8.14 (s, 1H), 7.92 (bs, 1H), 7.61 (dd,  $J = 4.3, 0.9$  Hz, 1H), 7.39 (m, 2H), 7.24 (m, 8H), 6.77 (m, 4H), 6.47 (t,  $J = 6.2$  Hz, 1H), 4.79 (m, 1H), 4.13 (m, 1H), 3.76 (s, 3H), 3.75 (s, 3H), 3.44 (dd,  $J = 10.23, 5.8$  Hz, 1H), 3.38 (dd,  $J = 10.23, 4.4$  Hz, 1H), 2.91 (m, 1H), 2.60 (m, 1H), 2.30 (m, 1H), 1.27 (m, 6H).

(E) Synthesis of 2-isobutyrylamino-6-(2-thienyl)-9-[2-deoxy-3-O-[(diisopropylamino)-(2-cyanoethoxy)] phosphino-5-O-dimethoxytrityl]- $\beta$ -D-ribofuranosyl purine (22)

The compound (212) 125 mg (0.18 mmol) obtained in the above (D) was azeotropically distilled three times with anhydrous pyridine 0.5 ml and azeotropically distilled three times with anhydrous tetrahydrofuran 0.5 ml. The residue was dissolved in anhydrous tetrahydrofuran 1.2 ml under argon

atmosphere, then added further diisopropylethylamine 46 ml and (2-cyanoethoxy) (N,N-diisopropylamino) phosphine chloride 59 ml and stirred at room temperature for 1 hour. Remained chloride was decomposed by adding methanol 50 ml. Ethyl acetate containing 3% triethylamine 25 ml was added to the reaction mixture, and water 25 ml was added for three times separation to obtain organic layer. Each aqueous layer was washed with 3% triethylamine containing ethyl acetate. The organic layer was collected, dried with anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by using short column (developer: 3% triethylamine - 32% ethyl acetate - 65% hexane) to obtain the product (22) 139 mg (0.16 mmol) (92.2%).

<sup>1</sup>H-NMR (270 MHz, CDCl<sub>3</sub>)  $\delta$ : 8.64 (m, 1H), 8.16 (m, 1H), 7.86 (m, 1H), 7.61 (m, 1H), 7.26 (m, 2H), 7.24 (m, 8H), 6.78 (m, 4H), 6.45 (m, 1H), 4.75 (m, 1H), 4.23 (m, 1H), 3.75 (m, 6H), 3.70 (m, 4H), 3.36 (m, 2H), 2.75 (m, 2H), 2.62 (m, 1H), 2.48 (m, 1H), 1.95 (m, 1H), 1.18 (m, 18H).

<sup>31</sup>P-NMR (270 MHz, CDCl<sub>3</sub>): 149.51, 148.43 ppm.

### Example 3:

Synthesis of 3-(2'-deoxy-5'-O-triphosphoryl-  $\beta$  -D- ribofuranosyl) pyridine-2-one (dYTP) (23) (refer to Fig. 4)

(A) Synthesis of 3-(3',5'-O-tetraisopropylidisiloxanyl-  $\beta$  -D- ribofuranosyl) pyridine-2-one (12)

3-( $\beta$ -D- ribofuranosyl) pyridine-2-one (11) [J. Matulic-Adamic and L. Beigelman, Tetrahedron Lett., 38, 203-206 (1997)] (2.29 mmol, 520 mg) was azeotropically dehydrated three times with anhydrous pyridine and was

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dissolved in anhydrous pyridine (23 ml). 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane (2.52 mmol, 0.81 ml) was added with stirring at room temperature and further stirred at room temperature for overnight. After confirming completion of the reaction by TLC, water was added to the reaction mixture to terminate the reaction and concentrated in vacuo. The residue was dissolved in chloroform. The organic layer was washed twice with aqueous 5% sodium hydrogen carbonate and once with aqueous saturated sodium chloride solution. The organic layer was dried with magnesium sulfate, and filtered. The filtrate was concentrated in vacuo. The residue was purified by using silica-gel chromatography (dichloromethane - methanol) to obtain the product (12) 442 mg (0.94 mmol) (41%).

<sup>1</sup>H-NMR (270.06 MHz, CDCl<sub>3</sub>) δ : 13.07 (br, 1H, NH), 7.78 (d, 1H, H4, J = 6.8 Hz), 7.37 (d, 1H, H6, J = 4.6 Hz), 6.29 (t, 1H, H5, J = 6.6 Hz), 5.07 (s, 1H, H1'), 4.01-4.30 (m, 5H, H2', H3', H4', H5', H5''), 0.83-1.10 (m, 28H, iPr).

(B)      Synthesis                      of                      3-(2'-O-imidazothiocarbonyl-3',5'-O-tetraisopropylidisiloxanyl-β-ribofuranosyl) pyridine-2-one (13)

The compound (12) (0.94 mmol, 442 mg) obtained in the above (A) was dehydrated azeotropically three times with anhydrous toluene, dissolved in anhydrous DMF (9 ml). Thiocarbonylimidazolide (2.24 mmol, 401 mg) was added under stirring at room temperature, then the reaction mixture was stirred at room temperature for 7 hours. After confirming completion of the reaction by TLC, ethyl acetate was added to the reaction mixture. The organic layer was washed twice with water, dried with magnesium sulfate, and the filtrate was concentrated in vacuo. The residue was purified by using silica-gel

column chromatography (dichloromethane - methanol) to obtain the product (13) 434 mg (0.749 mmol) (80%).

$^1\text{H-NMR}$  (270.06 MHz,  $\text{CDCl}_3$ )  $\delta$ : 13.40 (br, 1H, NH), 8.44 (s, 1H, imidazolidine), 7.84 (d, 1H, H4,  $J = 6.8$  Hz), 7.73 (s, 1H, imidazolidine), 7.33 (d, 1H, H6,  $J = 6.5$  Hz), 7.07 (s, 1H, imidazolidine), 6.34 (t, 1H, H5,  $J = 6.8$  Hz), 6.23 (d, 1H, H2',  $J = 5.1$  Hz), 5.25 (s, 1H, H1'), 4.46-4.52 (m, 1H, H3'), 4.25-4.29 (m, 1H, H5'), 4.03-4.09 (m, 2H, H4', H5''), 0.87-1.09 (m, 28H, iPr).

(C) Synthesis of 3-(2'-deoxy-3',5'-O-tetraisopropylidisiloxanyl-  $\beta$  - ribofuranosyl) pyridine-2-one (14)

The compound (13) (0.749 mmol, 434 mg) obtained in the above (B) was dehydrated azeotropically three times with anhydrous toluene, added ammonium sulfate (8.4 mg), dissolved in hexamethyldisilazane (12.6 ml) and refluxed for 1 hour. The reaction mixture was concentrated in vacuo, dehydrated azeotropically three times with anhydrous toluene, added azobisisobutyronitrile (83.5 mg) and dissolved in anhydrous toluene (16.8 ml). Tributyltin hydride (0.821 ml) was added to the reaction mixture and refluxed for 1 hour. After confirming completion of the reaction by TLC, the reaction mixture was concentrated in vacuo. The residue was purified by using silica-gel column chromatography (dichloromethane - methanol) to obtain the product (14) 0.268 g (0.591 mmol) (79%).

$^1\text{H-NMR}$  (270.06 MHz,  $\text{CDCl}_3$ )  $\delta$ : 13.07 (br, 1H, NH), 7.72 (d, 1H, H4,  $J = 7.0$  Hz), 7.31 (d, 1H, H6,  $J = 6.5$  Hz), 6.29 (t, 1H, H5,  $J = 6.6$  Hz), 5.20-5.25 (m, 1H, H1'), 4.37-4.40 (m, 1H, H3'), 3.97-4.12 (m, 2H, H5', H5''), 3.80-3.84 (m, 1H, H4'), 2.26-2.36 (m, 1H, H2'), 1.77-1.86 (m, 1H, H2''), 0.90-1.09 (m, 28H, iPr).

(D) Synthesis of 3-(2'-deoxy- $\beta$ -D-ribofuranosyl) pyridine-2-one (15)

The compound (14) (0.089 mmol, 42 mg) obtained in the above (C) was dehydrated azeotropically three times with anhydrous toluene, added 1 M tetramethyl ammoniumfluoride/THF solution (0.5 ml) and stirred at room temperature for 2 hours. After confirming completion of the reaction by TLC, acetic acid (0.08 ml) was added thereto and concentrated in vacuo. The residue was dissolved in water, washed three times with ethyl acetate, and the aqueous layer was concentrated in vacuo. The residue was purified by using reverse phase silica-gel chromatography to obtain the product (15) 0.14 mg (0.047 mmol) (52%).

$^1\text{H-NMR}$  (270.06 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.77 (d, 1H, H4,  $J = 3.8$  Hz), 7.36 (d, 1H, H6,  $J = 3.5$  Hz), 6.41 (t, 1H, H5,  $J = 3.6$  Hz), 5.01-5.17 (m, 1H, H1'), 4.29-4.31 (m, 1H, H3'), 3.93-3.95 (m, 1H, H4'), 3.62-3.70 (m, 2H, H5', H5''), 2.31-2.35 (m, 1H, H2'), 1.89-1.95 (m, 1H, H2'').

(E) Synthesis of 3-(2'-deoxy-5'-O-triphosphoryl-  $\beta$  -D-ribofuranosyl) pyridine-2-one (16)

The compound (15) (0.059 mmol, 13.4 mg) obtained in the above (D) was dehydrated azeotropically three times with anhydrous toluene, dissolved in trimethyl phosphate (0.2 ml), added phosphorus oxychloride (0.065 mmol, 7.1  $\mu\text{l}$ ) under ice-cooling and stirred for 7 hours under ice-cooling. After confirming completion of the reaction by TLC, well mixed solution of 0.5 M bis(tributylammonium) pyrophosphate-DMF solution and tributylamine (70.2  $\mu\text{l}$ ) was immediately added and stirred well under ice-cooling for 30 minutes. 1

M triethylammonium bicarbonate (0.35 ml) was added to the reaction mixture to terminate the reaction and concentrated in vacuo. The residue was dissolved in water and charged on a column of DEAE-Sephadex chromatography (15 × 300 mm) and eluted by gradient elution with 50 mM - 1 M triethylammonium bicarbonate. A fraction eluted at 0.53 - 0.59 M was collected and lyophilized. Structure was confirmed by MS(ESI-), <sup>1</sup>H-NMR and <sup>31</sup>P-NMR. Sodium salt was prepared by treating with Dowex 50Wx8 column chromatography.

MS(ESI-): (M-H<sup>-</sup>) 449.9.

<sup>1</sup>H-NMR (270.06 MHz, CDCl<sub>3</sub>) δ : 7.83 (d, 1H, H4, J = 4.9 Hz), 7.35 (d, 1H, H6, J = 4.9 Hz), 6.51 (t, 1H, H5, J = 4.9 Hz), 5.17 (t, 1H, H1', J = 5.0 Hz), 4.56 (br, 1H, H3'), 4.06 (br, 1H, H4'), 3.99 (br, 2H, H5', H5''), 2.19-2.33 (m, 1H, H2'), 1.81-1.98 (m, 1H, H2'').

<sup>31</sup>P-NMR (109.36 MHz, D<sub>2</sub>O) δ : -10.3 (m, 2P, P<sup>1</sup>, P<sup>3</sup>), -22.7 (m, 1P, P<sup>2</sup>).

UV (10 mM phosphate buffer pH 7.0): λ<sub>max</sub> = 298 nm (ε = 7.6 × 10<sup>3</sup>), 226 nm (ε = 7.0 × 10<sup>3</sup>), λ<sub>min</sub> = 247 nm, 211 nm.

#### Example 4:

##### Synthesis of primer and template

Following primer and template were synthesized conventionally by using DNA/RNA synthesizer Type 392, The Perkin-Elmer, Applied Biosystems Div., and cyanoethylamidite reagents of dA, dC, dG and dT, which were available from The Perkin-Elmer, and dX of cyanoethylamidite reagent hereinbefore.

Proviso that in a synthesis of oligomer containing dX, removal of protective group for amino group of dX, i.e. benzoyl group, could not completely

be performed by conventional condition using conc. ammonia at 55°C for overnight, consequently, treatment for removal of the protective group was performed under the condition at 80°C with conc. ammonia for 10 hours.

Primer 1: dgcaciacctatagg

Primer 2: dcataggaggaga

Primer 3: dgcctagtgtgaccg

Template 1: dtgctctatcttctccctatagtgtgagtcgtattat

Template 2: dtgctctgtcttctccctatagtgtgagtcgtattat

Template 3: dtgctctxtcttctccctatagtgtgagtcgtattat

Template 4: dagctgtgtgtgtctccgggtacaactaggc

Template 5: dagctxtgtgtgtctccgggtacaactaggc

Template 6: dagctxxgtgtgtctccgggtacaactaggc

Template 7: dagctxtxtgtgtctccgggtacaactaggc

Template 8: dagctxtgxtgtgtctccgggtacaactaggc

Template 9: dagctxtgtxtgtgtctccgggtacaactaggc

#### Example 5:

##### 5'-<sup>32</sup>P labeling of primer

Primer 1-4 (ca. 1 nmol), 10x polynucleotide kinase buffer (TAKARA) 2  $\mu$ l, [ $\gamma$ -<sup>32</sup>P]-dATP (ca. 1.1 TBq/mmol) 2  $\mu$ l, and polynucleotide kinase (10 unit/ $\mu$ l, TAKARA) 2  $\mu$ l were added into a tube 0.5 ml. The mixture, total 20  $\mu$ l, was incubated at 37°C for 40 minutes. The reaction was terminated by adding 10 M urea BPB dye 10  $\mu$ l, treated at 75°C for 5 minutes, then electrophoresed using 20% polyacrylamide 7M urea gel electrophoresis (10 cm



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× 10cm). Main band detected by UV (254 nm) was cut out, transferred to 1.5 ml tube, adding 450  $\mu$ l of sterilized water and stirred at 37°C for 12 hours. Supernatant obtained by light centrifugation was transferred to the different tube, added glycogen 1  $\mu$ l, 3M sodium acetate 40  $\mu$ l and ethanol 1 ml were added. The mixture was shaken well, thereafter allowed to stand at -30°C for 1 hour. Then it was centrifuged at -5°C, under 13,000 rpm for 1 hours. The thus obtained precipitate was rinsed with 70% ethanol and dried by using centrifugal evaporator for 30 minutes. Sterilized water 40  $\mu$ l was added and kept at 75°C for 5 minutes, thereafter quantitated at UV 260 nm.

Example 6:

Single nucleotide insertion reaction and primer extension reaction using Klenow fragment

5'-<sup>32</sup>P labeled primer, template and 10 x Klenow fragment buffer (TAKARA) 1  $\mu$ l were added to the 0.5 ml tube, adjusted total volume to 7  $\mu$ l, and annealed at 95°C for 3 minutes, at 40°C for 3 minutes and at 4°C for 7 minutes. dNTP 1  $\mu$ l, Klenow fragment (1 unit/ml, For Sequencing, TAKARA) 2  $\mu$ l were added, adjusted to total volume to 10  $\mu$ l and incubated for the fixed time at 17°C. The reaction was terminated by adding 10 M urea BPB dye 5  $\mu$ l, heated at 75°C for 5 minutes, and electrophoresed with 20% polyacrylamide gel with 7M urea gel electrophoresis. The result was analyzed using imaging plates (Phosphorimager analysis). Results are shown in Fig. 6, Fig. 7 and Fig. 9. Single nucleotide insertion reaction is shown in Fig. 7 and primer extension reaction is shown in Fig. 6 and Fig. 9, respectively.

Example 7:

Inhibition experiment for primer extension reaction using Klenow fragment

Primer, template and 10x Klenow fragment buffer (TAKARA) 1  $\mu$ l were added to 0.5 ml tube, and total volume was adjusted to 7  $\mu$ l, and annealed at 95°C for 3 minutes, at 40°C for 3 minutes and at 4°C for 7 minutes. [ $\alpha$ -<sup>32</sup>P] TTP or [ $\alpha$ -<sup>32</sup>P] dCTP and dYTP were added to final concentration for each level, and Klenow fragment (1 unit/ml, For Sequencing, TAKARA) 2  $\mu$ l was added, adjusted to total volume to 10  $\mu$ l and incubated for the fixed time at 17°C. The reaction was terminated by adding 10 M urea BPB dye 5  $\mu$ l, kept at 75°C for 5 minutes, and electrophoresed with 20% polyacrylamide gel with 7M urea gel electrophoresis. The result was analyzed using imaging plates (Phosphorimager analysis). Result is shown in Fig. 8.

Example 8:

Transcription by T7 RNA polymerase

Template DNA 1  $\mu$ M, in which promoter region has duplicated strands, and T7 RNA polymerase 2.5 units were added to a solution containing 2mM rNTP, [ $\alpha$ -<sup>32</sup>P] ATP 0.1  $\mu$ Ci/ $\mu$ l [40 mM Tris-HCl (pH 8.0), 8 mM MgCl<sub>2</sub>, 2 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 10 mM rGMP], and incubated for 3 hours. After the reaction, 10 M urea dye was added and kept at 75°C for 3 minutes, then electrophoresed with 20% polyacrylamide gel. The product was analyzed. Result is shown in Fig. 10.

Example 9:

Transcription using T7 RNA polymerase

Reaction was performed as same as in example 8. The generated RNA was isolated by gel electrophoresis. RNA was digested by 0.75 units RNase T2. Each nucleotide was separated using 2-dimension TLC and each ratio was calculated.

Result is shown in Fig. 11. Ratio of composition of each nucleotide is shown in Table 1 hereinbefore.

Example 10:

Single nucleotide insertion reaction using Klenow fragment (exo<sup>+</sup>)

A solution containing [5'-<sup>32</sup>P] labeled primer DNA (20-mer, 4 mM), template DNA (35-mer, 4 mM) and 2x Klenow fragment buffer (TAKARA) were annealed at 95°C for 3 minutes, 40°C for 3 minutes and 4°C for 7 minutes. A solution of equimolar amount of 40 mM dNTP and Klenow fragment (exo<sup>+</sup>) (2 unit/ml, For Sequencing, TAKARA) were added thereto and incubated at 37°C for 3 minutes. Equimolar amount of 10 M urea BPB dye solution was added and kept at 75°C for 5 minutes and electrophoresed with 20% polyacrylamide - 7M urea gel. Products were analyzed by using Phosphorimager plate. Result is shown in Fig. 12.

Example 11:

Transcription by T7 RNA polymerase

A solution containing template DNA 1mM, in which promoter region has duplicated strands, T7 RNA polymerase 2.5 units, 2mM rNTP, and [ $\alpha$ -<sup>32</sup>P]

rATP 0.1mCi/ml [40 mM Tris-HCl (pH 8.0), 8 mM  $MgCl_2$ , 2 mM spermidine, 5 mM DTT, 0.01% Triton X-100, 10 mM rGMP] were prepared and incubated for 3 hours. 10 M urea dye was added and kept at 75°C for 3 minutes to terminate the reaction. The product RNA (16-mer) in this solution was purified by using electrophoresis with 20% polyacrylamide gel. RNA was digested by 0.75 units RNase T2. Ratio of each nucleotide was determined by 2-dimension TLC (cellulose resin). In Fig. 13, result of development of TLC is shown. Ratio of each nucleotide is shown in Table 2 hereinbefore.

#### Example 12:

##### Synthesis of primer and template containing base X2

Primer and template were synthesized conventionally by using DNA/RNA synthesizer Type 392, The Perkin-Elmer, Applied Biosystems Div., and cyanoethylamidite reagents of dA, dC, dG and dT, which were distributed by The Perkin-Elmer, and dx2 of cyanoethylamidite reagent prepared according to the method in example 1.

Proviso that in a synthesis of oligomer containing dx2, removal of protective group for amino group of dx2, i.e. benzoyl group, could not completely be performed, under the conventional condition using conc. ammonia at 55°C for overnight, consequently, treatment for removal of the protective group was performed under the condition at 80°C with conc. ammonia for 10 hours.

#### Industrial Applicability

The present invention indicates that selective base pair formation,

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which could never achieved by the heretofore reported artificial base pair, can be realized by utilizing steric hindrance and electrostatic repulsion as well as stacking action. By utilizing the method of the present invention, artificial nucleic acid base pair of the present invention can be applied on replication and transcription of nucleic acid, and protein synthetic system or functional nucleic acid. For example, by using artificial base pair of the present invention, in vitro selection method used by the natural base of 4 types can be performed by 6 types of bases. Creation of nucleic acid molecules having new function which can not be realized by 4 natural bases. Further, novel base pair of the present invention may be utilized for treatment of hereditary diseases caused by replacement of one or more bases to the other bases.

## CLAIMS

1. A method for constructing selective base pair comprising introducing a group having ability to form steric hindrance between bases in nucleic acid base.
2. The method according to claim 1 wherein the group having ability to form steric hindrance is a group to hinder formation of base pair with base part of natural nucleic acid.
3. The method according to claim 1 or claim 2 wherein the group having ability to form steric hindrance is dialkylamino group.
4. A method for constructing selective base pair comprising introducing a group having ability to form steric hindrance and electrostatic repulsion, and stacking action between bases in nucleic acid base.
5. The method according to claim 4 wherein the group having ability to form steric hindrance and electrostatic repulsion, and stacking action is a group to hinder formation of base pair with base part of natural nucleic acid.
6. The method according to claim 4 or claim 5 wherein the group having ability to form steric hindrance and electrostatic repulsion, and stacking action is aromatic heterocyclic group.
7. The method according to claim 6 wherein aromatic heterocyclic group is five membered or six membered aromatic heterocyclic group having 1 or 2 sulfur atoms, oxygen atoms or nitrogen atoms as a heteroatom.
8. The method according to claim 6 or claim 7 wherein aromatic heterocyclic group is thiophene.
9. The method according to any of claims 1 - 8 comprising introducing a

group to be able to for additional hydrogen bonds.

10. The group to be able to for additional hydrogen bond is an electron pair of amino group, hydroxyl group, keto group or nitrogen group.

11. The method according to any of claims 1 - 10 wherein the base pair is a base pair which can be recognized by polymerase.

12. The method according to claim 11 wherein the polymerase is DNA polymerase or RNA polymerase.

13. A method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance in the nucleic acid base part.

14. A method for designing nucleic acid to construct selective base pair comprising hindering to construct base pair with the natural nucleic acid base part by utilizing the steric hindrance.

15. A method for designing nucleic acid to construct selective base pair comprising utilizing steric hindrance and electrostatic repulsion, and stacking action in the nucleic acid base part.

16. A method for designing nucleic acid to construct selective base pair comprising hindering to construct base pair with the natural nucleic acid base part by utilizing steric hindrance and electrostatic repulsion and stabilizing to impart stacking action.

17. The method for designing nucleic acid according to any of claims 13 - 16 wherein the nucleic acid base pair is a base pair which can be recognized by polymerase.

18. A nucleic acid comprising being designed by the method according to any of claims 13 - 17.

19. The nucleic acid according to claim 18 wherein the nucleic acid has

base comprising purine derivatives having a group at position-6 which can generate steric hindrance.

20. The nucleic acid according to claim 19 wherein the nucleic acid base is 2-amino-6-N,N-dimethylamino-purine.

21. The nucleic acid according to claim 19 wherein the nucleic acid base is 2-amino-6-thienyl-purine or derivatives thereof.

22. The nucleic acid according to claim 18 wherein the nucleic acid has base containing pyridine having hydroxyl group or keto group at position-2.

23. The nucleic acid according to claim 22 wherein the nucleic acid base is pyridine-2-one or tautomer thereof.

24. The nucleic acid according to any of claims 18 - 23 wherein the nucleic acid is a nucleic acid constructing base pair with its complementary nucleic acid.

25. A process for production of the nucleic acid comprising the nucleic acid according to any of claims 18 - 23.

26. The process according to claim 25 wherein the nucleic acid is another nucleic acid to construct base pair.

27. A codon comprising one or more nucleic acid according to any of claims 18 -24.

28. The codon according to claim 27 wherein the codon encodes amino acids.

29. The codon according to claim 28 wherein amino acids are non-natural amino acids.

30. A nucleic acid molecule comprising containing the nucleic acid according to any of claims 18 - 24 and natural nucleic acid.



31. The nucleic acid molecule according to claim 30 wherein the nucleic acid molecule encodes proteins.
32. The nucleic acid molecule according to claim 30 or claim 31 wherein the nucleic acid molecule has whole or part of genetic information of the natural gene.
33. A process for production of nucleic acid having complementary strand thereof comprising reacting the polymerase with the nucleic acid according to any of claims 30 -32.
34. The method according to claim 33 wherein the polymerase is DNA polymerase or RNA polymerase.
35. A process for production of non-natural nucleic acid comprising inserting or substituting one or more nucleic acid according to any of claims 18 - 24 in the natural nucleic acid.
36. The process for production of non-natural nucleic acid according to claim 35 wherein a position, to which the nucleic acid according to any of claims 18 - 24 is inserted or substituted, has a codon unit and the other part has base sequence encoding natural amino acid sequence.
37. A process for production of protein having amino acid sequence based on codons of the nucleic acid according to any of claims 30 -32 or the non-natural nucleic acid obtained by the method according to claim 35 or claim 36.
38. The process for production of protein according to claim 37 comprising being inserted or substituted by the non-natural amino acid in the part or whole of amino acid sequence of natural protein.
39. A microorganism which is transformed by non-natural gene which can be produced by the process according to claim 35 or claim 36.

40. A method for screening functions of amino acids coded by natural gene comprising using the non-natural gene which can be produced by the method according to claim 35 or claim 35.

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Fig. 1

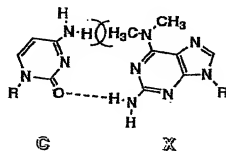
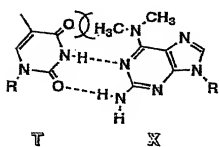
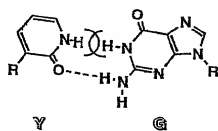
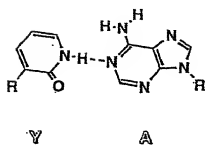
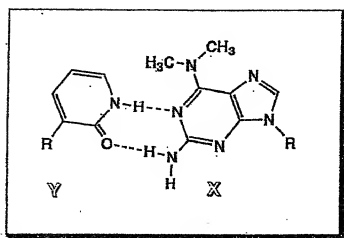
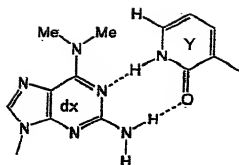
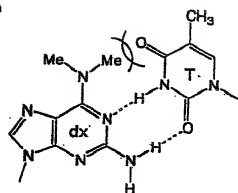


Fig. 2

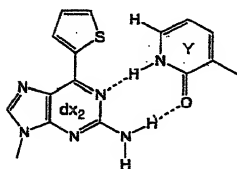
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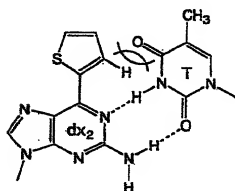
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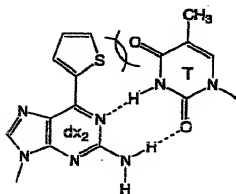
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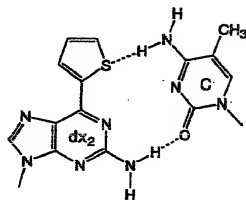
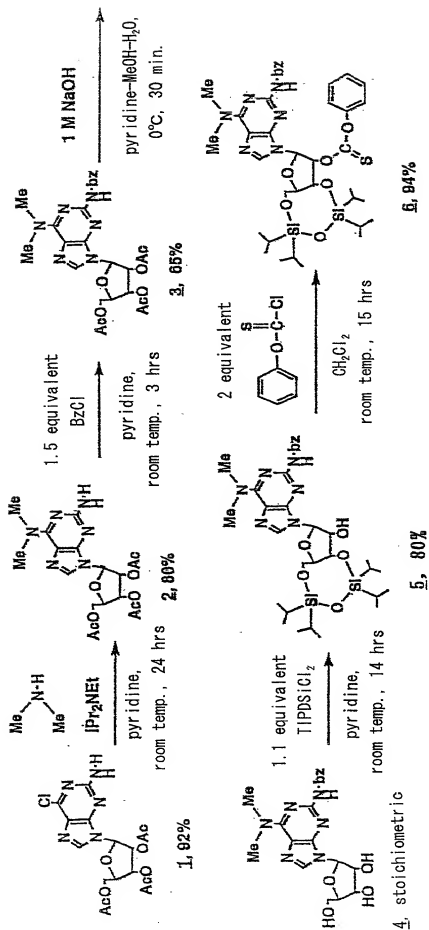
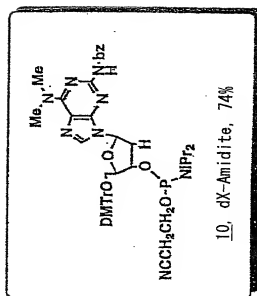


Fig. 3





10, dX-Amidite, 74%

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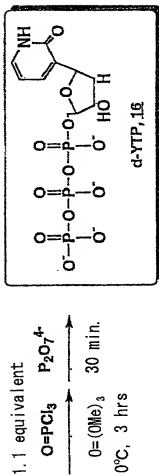
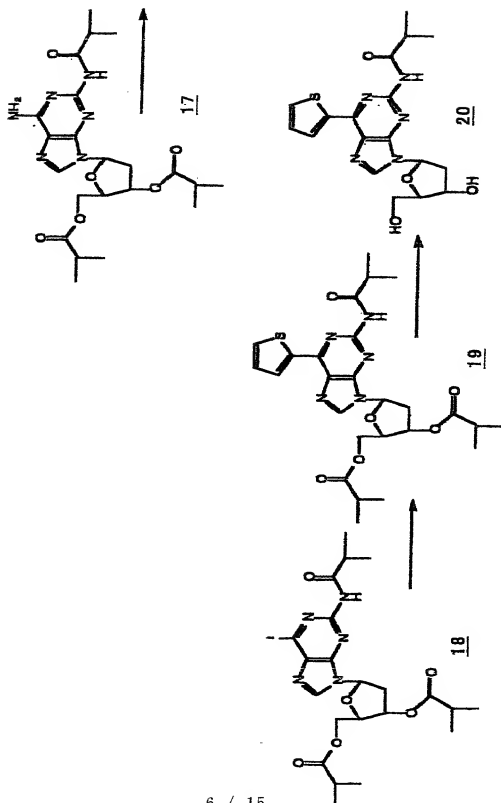
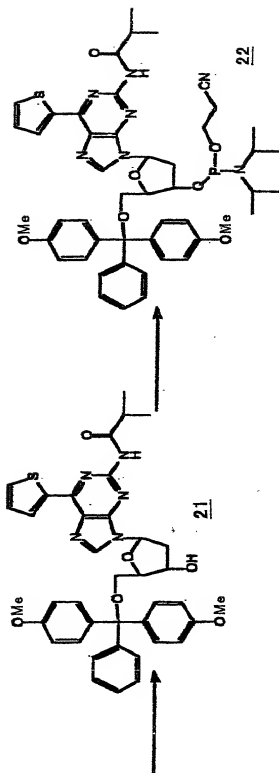


Fig. 5







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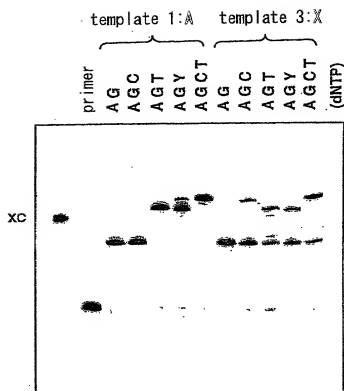


Fig. 7

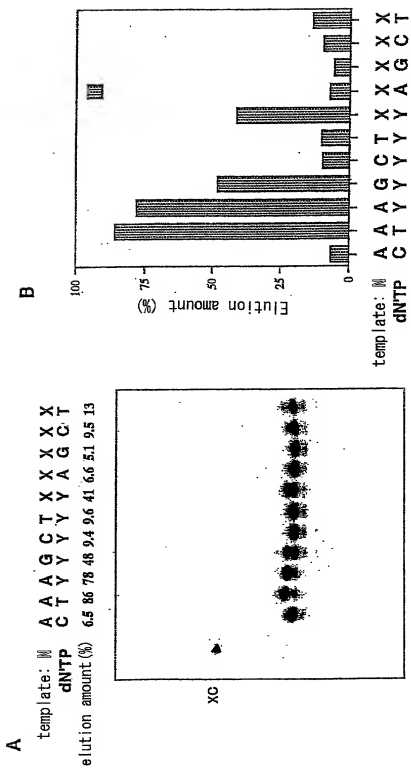


Fig. 8

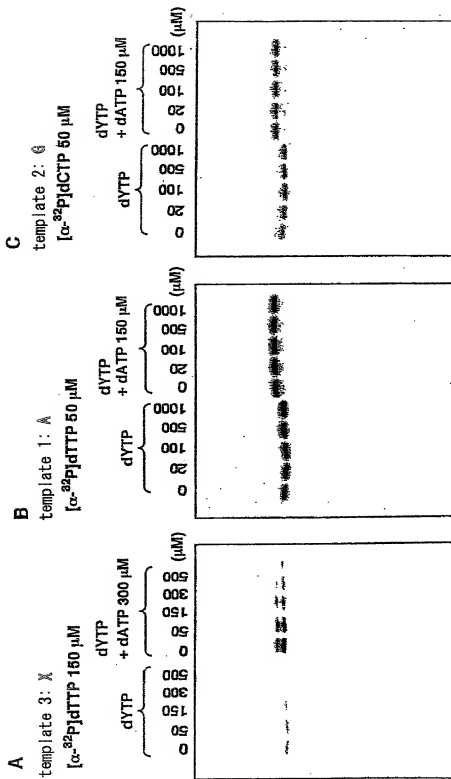


Fig. 9

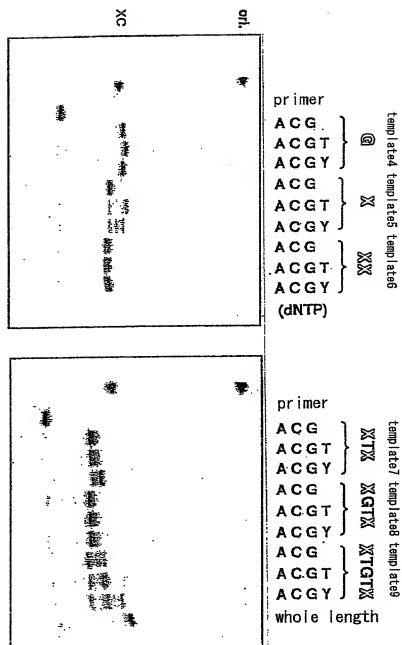


Fig. 10

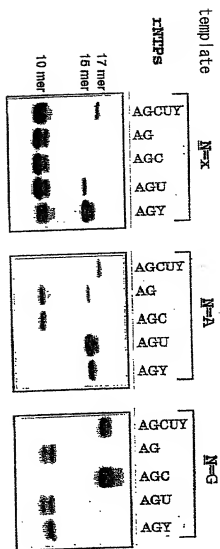
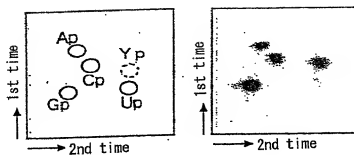
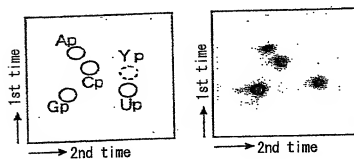


Fig. 11

A



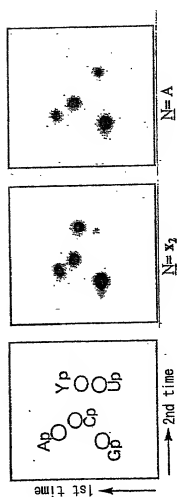
B



[illegible]



Fig. 13



**Declaration and Power of Attorney for Patent Application**  
**English Language Declaration**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**NOVEL NUCLEIC ACID BASE PAIR**

the specification of which

(check one)

- ☒ corresponds to and claims priority of Japanese Patent Application No. 11-201450/1999, ✓  
filed July 15, 1999, Japanese Patent Application No. 2000-133519, filed May 2, 2000 and ✓  
PCT/JPO0/04720, filed July 14, 2000 ✓
- ☐ was filed on \_\_\_\_\_ as United States Application No. or PCT  
Application No. \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

<u>11-201450/1999</u> (Number)	<u>Japan</u> (Country)	<u>15 July 1999</u> ✓ (Day/Month/Year Filed)	[ ]
<u>2000-133519</u> (Number)	<u>Japan</u> (Country)	<u>2 May 2000</u> ✓ (Day/Month/Year Filed)	[ ]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	[ ]

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

I hereby claim the benefit under 35 U.S.C. Section 120 of the United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark office all information known to me to be material to patentability as defined in Title 37, C.F.C., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/JP00/04720 ✓

\_\_\_\_\_  
(Application Serial No.)

July 14, 2000 ✓

\_\_\_\_\_  
(Filing Date)

Pending

\_\_\_\_\_  
(Status)

(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

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(Status)

(patented, pending, abandoned)

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(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)

(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

00707136:042604

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*list name and registration number*)

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 Cara Z. Lowen Reg. No. 38,227  
 William J. Daley, Jr. Reg. No. 35,487  
 Steven M. Jensen Reg. No. 42,693

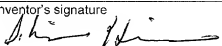
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3-00

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4-00

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Citizenship	
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## SEQUENCE LISTING

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